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ROLE OF THE PILUS IN F PLASMID-MEDIATED BACTERIAL CONJUGATION

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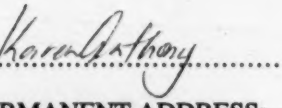

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Abstract

The conjugation system encoded by F-like plasmids is dependent on the elaboration of an extracellular pilus. In addition to providing receptor sites for the attachment of pilus-specific bacteriophages, the pilus has been implicated in recipient cell recognition and surface exclusion. However, neither the molecular mechanism nor the complementary role of the pilus in these processes has been resolved.

In the present study, a systematic genetic analysis of the gene products involved in pilus synthesis was carried out to understand the molecular basis for the specificity in mating-pair formation and surface exclusion among F-like plasmids. Complementation analysis of a *traA* null mutant with the homologous alleles from ColB2 and R100-1 plasmids revealed that the pilin sequence does not govern the specificity in recognition of LPS receptors or TraT in recipient cells. Furthermore, with the exception of TraG, none of the gene products involved in pilus synthesis determined the specificity of pilus function. Complementation analysis of an F *traG* mutant with the corresponding allele from R100-1 resulted in plasmid transfer to recipient cells expressing the F TraT and TraS proteins without any significant surface exclusion. Thus, the surface exclusion specificity among F-like plasmids is determined by the allelic differences in *traG*. Together with the observation that requirements for specific LPS moieties and OmpA in recipient cells for F plasmid transfer, is determined by the TraN protein, these results reveal that specificities in recipient cell recognition and surface exclusion are brought about by the mating-pair stabilization system and not by the pilus.

To understand the basis for the variation in the plating efficiencies of f1 phage among F-like plasmids, studies were undertaken to characterize the nature of the F pilus tip. Using a fusion protein comprising the pilus attachment domain of the pIII protein of M13 phage, the presence of an adhesin at the pilus tip was investigated. Lack of a distinct tip protein or other minor proteins associated with the F pilus, suggested a unique arrangement of pilin subunits at the tip. Further, indirect evidence implicates conformational differences among F-like pili as the basis for variation in susceptibility to pilus-specific phages.

To The Memory of

My

MOTHER

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Amperes
Å	Angstrom
aa	Amino acid. IUPEC notations used
Amp	Ampicillin
AP	Alkaline phosphatase
APS	Ammonium persulfate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
bp	base pair
CAT	Chloramphenicol acetyl transferase
Cm	Chloramphenicol
Da	Dalton
DNA	Deoxyribonucleic acid
ds	double-stranded
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
g	gram
Glc	Glucose
hr	Hour
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
Inc	Incompatibility
IPTG	Isopropyl ß-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
l	litre
LB	Luria-Bertani medium
M	Molar
mAb	Monoclonal antibody
mCi	MilliCurie
min	Minute
mM	Milli molar (unit prefix = 10^{-3})
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
nmol	Nano mole (unit prefix = 10^{-9})
Ni^{+}-NTA	Nickel nitrilotriacetate
OD_{600}	Optical density at 600 nm wavelength

ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	Plaque forming units
pg	Picogram (unit prefix = 10^{-12})
pmol	Picomole (unit prefix = 10^{-12})
PVDF	Polyvinylidene difluoride
^{R}	Resistant
rpm	Revolutions per minute
RT	Room temperature
RBS	Ribosome binding site
RNA	ribonucleic acid
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
Sfx	Surface exclusion index
Sm	Streptomycin
Sp	Spectinomycin
ss	Single-stranded
Tc	Tetracycline
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
Tn	Transposon
Tris	Tris(hydroxymethyl)aminomethane
TEM	Transmission electron microscopy
UV	Ultraviolet
V	Volts
w/v	Weight/volume
λ	bacteriophage lambda
µl	Microlitre
µm	Micrometre
°C	Degree Celsius
[γ^{32}P]-ATP	Gamma ^{32}phosphorous -adenosine triphosphate
[^{35}S]-methionine	^{35}Sulfur-methionine
ρ	Density
r	Refractive index

CHAPTER 1

Review of the literature

1.1 INTRODUCTION

One of the most important landmarks in the history of genetics is the discovery of a new kind of genetic element, the sex factor, which determines the ability of *Escherichia coli* K-12 to conjugate and transfer its genetic material to recipient cells (Hayes, 1968). Fifty years since its first discovery, bacterial conjugation, the process which promotes DNA transfer from a donor to a recipient cell upon physical contact, is known to be ubiquitous in nature. This phenomenon is not only responsible for the interspecies and intergeneric mobilization of DNA between Gram-negative and Gram-positive bacteria (Clewell, 1993), but also for the transmission of genetic material between kingdoms, as exemplified by the transfer of Ti plasmids from *Agrobacterium* species to plants (Kado, 1993) and the transfer of broad and narrow-host range plasmids from *E. coli* to *Saccharomyces cerevisiae* (Heinemann, 1991; Heinemann and Sprague, 1989). Thus, as a means of genetic exchange among individual cells and between bacterial species as well as between other kingdoms, bacterial conjugation is a process of fundamental evolutionary and ecological consequence (Firth *et al.*, 1996).

The fertility factor, F, of *E. coli* K-12 was the first conjugative plasmid to be discovered and studied extensively (Lederberg and Tatum, 1946). The knowledge and insights gained from these studies have helped elucidate a conserved mechanism of DNA transfer adopted by other conjugative plasmids in Gram-negative bacteria. This mechanism is dependent on the expression of the 'pilus', an extracellular filamentous structure essential for initiating contact with a recipient cell and mediate the subsequent transfer of DNA (Brinton, 1971; Curtiss, 1969). Although the pilus is a central feature in Gram-negative conjugation, the precise nature by which it facilitates this process is not understood. Indeed, all the biochemical and physical information gathered thus far reaffirms the importance of the pilus, but has not elucidated the role of the pilus in either recipient cell recognition or DNA transfer. Recent findings that the pilus assembly machinery shares sequence homology with the *ptl* operon of *Bordetella pertussis* involved in the export of exotoxin (Covacci and Rappuoli, 1993; Weiss *et al.*, 1993), suggests that the pilus may represent a highly specialized transport apparatus for the export of macromolecular complexes composed of DNA or protein.

Since the subject of this thesis is the F pilus, what ensues is a brief account of the history of conjugation followed by an analysis of the literature on F pilus including our knowledge on the biochemical and physical properties of the pilus as well as its role in conjugation and pilus-specific phage infection.

1.2 BACTERIAL CONJUGATION

1.2.1 A historical perspective

In 1946, Lederberg and Tatum discovered that the presence of the F fertility factor in donor cells enabled them to transmit their DNA unidirectionally to F⁻ cells in the population by a process they termed 'conjugation' (Lederberg and Tatum, 1946). F was later discovered to be either an episome or a plasmid (Jacob and Wollman, 1958) which was able to either replicate autonomously or stably integrate into the *E. coli* genome (Hfr) and mediate the efficient mobilization of chromosomal genes. The ordered transfer of chromosomal markers by Hfr donors led to the first genetic evidence for the circular nature of the *E. coli* chromosome and aided the development of a circular linkage map of *E. coli* K-12 (Hayes, 1968). The F plasmid has since been used to develop many of the techniques commonly used to dissect prokaryotic genetic systems and the analysis of its genetics and biochemistry has been central in elucidating the basic mechanisms of plasmid replication and transmission (Willetts and Skurray, 1987).

1.2.2 The F factor of *E. coli* K-12

Due to its constitutive nature of transfer, the F plasmid was responsible for the earliest observation of genetic transfer and has since remained a paradigm for all conjugative plasmids in Gram-negative bacteria (Lederberg and Tatum, 1946; Cavalli *et al.*, 1953). F is a 100 kb covalently closed double-stranded DNA molecule which encodes all the genetic elements necessary for its stable maintenance and transmission (Willetts and Skurray, 1987). Among these is a region designated as the 'Leading region' which is believed to be the first to enter the recipient cell during conjugation and its gene products are thought to assist in establishing F DNA in the recipient cell (Ray and Skurray, 1983). The autonomous

replication properties of F are encoded by the RepFIA sequences which are stringently regulated for maintaining the plasmid at one or two copies per cell (Willetts and Skurray, 1987). A 33.3 kb nucleotide sequence encodes the transfer (*tra*) operon which contains a series of gene products required for the conjugative transmission of the plasmid. The entire nucleotide sequence of the *tra* region has been determined and recently compiled (Frost *et al.*, 1994). A circularized 55 kb *Hin*dIII fragment which includes RepFIA as well as the *tra* and leading regions forms a mini-F plasmid, pOX38, whose derivatives serve as convenient tools for in-depth analyses of the transfer region (Guyer *et al.*, 1980). The F sequence also includes several insertion elements which promote the integration of the plasmid into the chromosome to give rise to the Hfr state. F prime (F') plasmids can be formed through imprecise excision resulting in the inclusion of chromosomal sequences on the F plasmid (Firth *et al.*, 1996). Interruption of the regulatory gene of conjugation, *finO*, by an IS3 element is responsible for the constitutive or the derepressed nature of F transfer (Cheah and Skurray, 1986).

1.2.3 Classification of other naturally occurring conjugative plasmids

Since the discovery of F, numerous other conjugative plasmids have been found throughout the family *Enterobacteriaceae* (Jacob *et al.*, 1977). These plasmids were classified into various incompatibility (Inc) groups based on the inability of a plasmid to coexist with another from the same Inc group due to sharing of replication proteins (Datta, 1975). Generally, plasmids within an Inc group exhibit similar plasmid size and *tra* gene organization and typically possess morphologically and serologically similar pili (Datta, 1975).

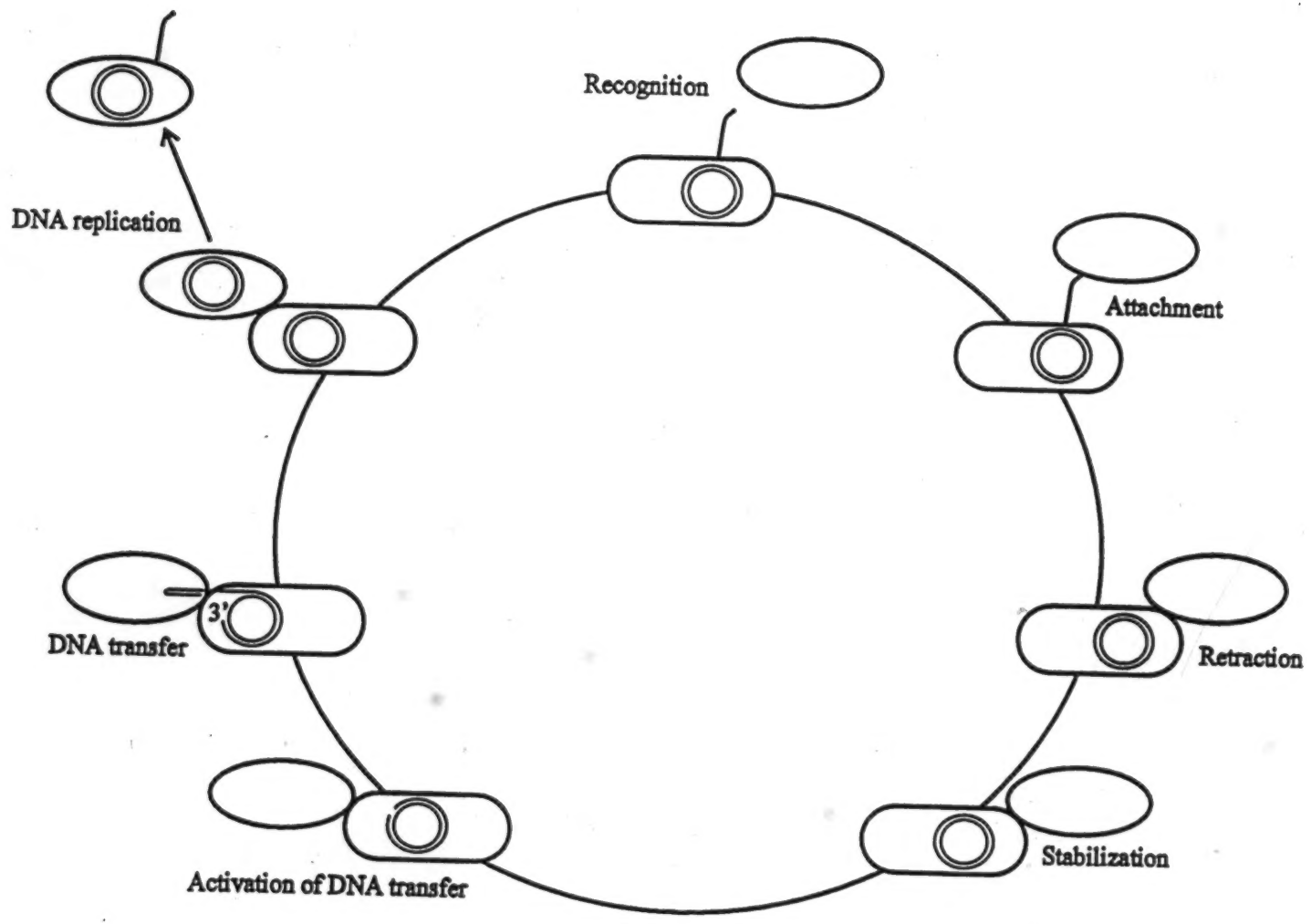
The IncF complex contains a large group of plasmids collectively known as 'F-like', which were initially classified into the F-like group based on the morphological and serological similarities of their pili and their ability to confer susceptibility to a group of pilus-specific bacteriophages (Datta, 1975; Lawn *et al.*, 1967). F-like plasmids belong to one of seven IncF incompatibility groups (IncFI-IncFVII) and they specify similar mating systems but differ from each other by subtle variations in their mating specificity, pilus

serology and pilus-specific bacteriophage adsorption and sensitivity (Lawn *et al.*, 1967). The basis of this variation will be discussed at length in later sections. Two F-like plasmids of the IncFII group which will be pertinent to this study, are the multiple drug resistant plasmid, R100-1, and the ColB2 (ColB2-K77) plasmid which encodes genes for colicin production (Sugino and Hirota, 1962; Hausmann and Clowes, 1971). R100-1 is the derepressed mutant of the 94.5 kb R100 plasmid also referred to as NR1 or 222 (Womble and Rownd, 1988). NR1 is the original bacterial resistance factor or the R factor isolated by Rintaro and Nakaya in Japan in the late 1950s and is the archetype of similar R factors isolated world-wide (Womble and Rownd, 1988). The R plasmids were extensively studied during the 1960s due to an alarming increase in the prevalence of antibiotic resistance among clinical isolates of bacterial pathogens brought about by their rapid self-transmission. Like F, the R100-1 plasmid exhibits high levels of constitutive transfer due to a mutation in *finO* gene (Yoshioka *et al.*, 1987). Genetic and physical analyses of NR1 revealed a gene organization very much similar to the F plasmid itself (Womble and Rownd, 1988). Heteroduplex mapping studies between F and the homologous R6-5 plasmid DNA further substantiated the high degree of homology between F and R100-1 *tra* regions (Sharp *et al.*, 1973), although subtle changes in their *tra* gene products which might address small differences between the transfer systems of the two plasmids are emerging from DNA sequence data currently being generated in our laboratory (Frost, unpublished data). The colicin-producing ColB2-K77 plasmid was first described by Hausmann and Clowes (1971) as having the ability to repress F transfer when coresident in a cell. Its derepressed form, ColB2Fdr, also known as pED236, is a 96 kb plasmid encoding a conjugative system that is almost identical to F as determined from comparison of available nucleotide sequences of its *tra* region (Willetts and Maule, 1986; Finlay *et al.*, 1984).

1.2.4 The conjugation process

The conjugation process mediated by the F plasmid can be divided into five orderly stages with events at each stage orchestrated by a specific set of *tra* proteins. The following are the stages that constitute the so-called 'mating cycle' illustrated in Fig.1.1 (Willetts and Skurray,

Fig. 1.1 The Mating Cycle. The diagram schematically depicts the basic steps involved in F plasmid-mediated conjugation. The process begins with the recognition of a recipient cell (shown as an elipse) by the pilus of a donor bacterium. Subsequent retraction of the pilus brings the recipient into closer wall-to-wall contact. Following stabilization of the mating pair, a single-strand of DNA is transferred into the recipient cell in 5'→3' direction. Concomitant to the transfer event, replacement and complementary strand synthesis occurs in the donor and recipient, respectively.


Recognition
Attachment
Retraction
Stabilization
Activation of DNA transfer
DNA transfer
3'
DNA replication

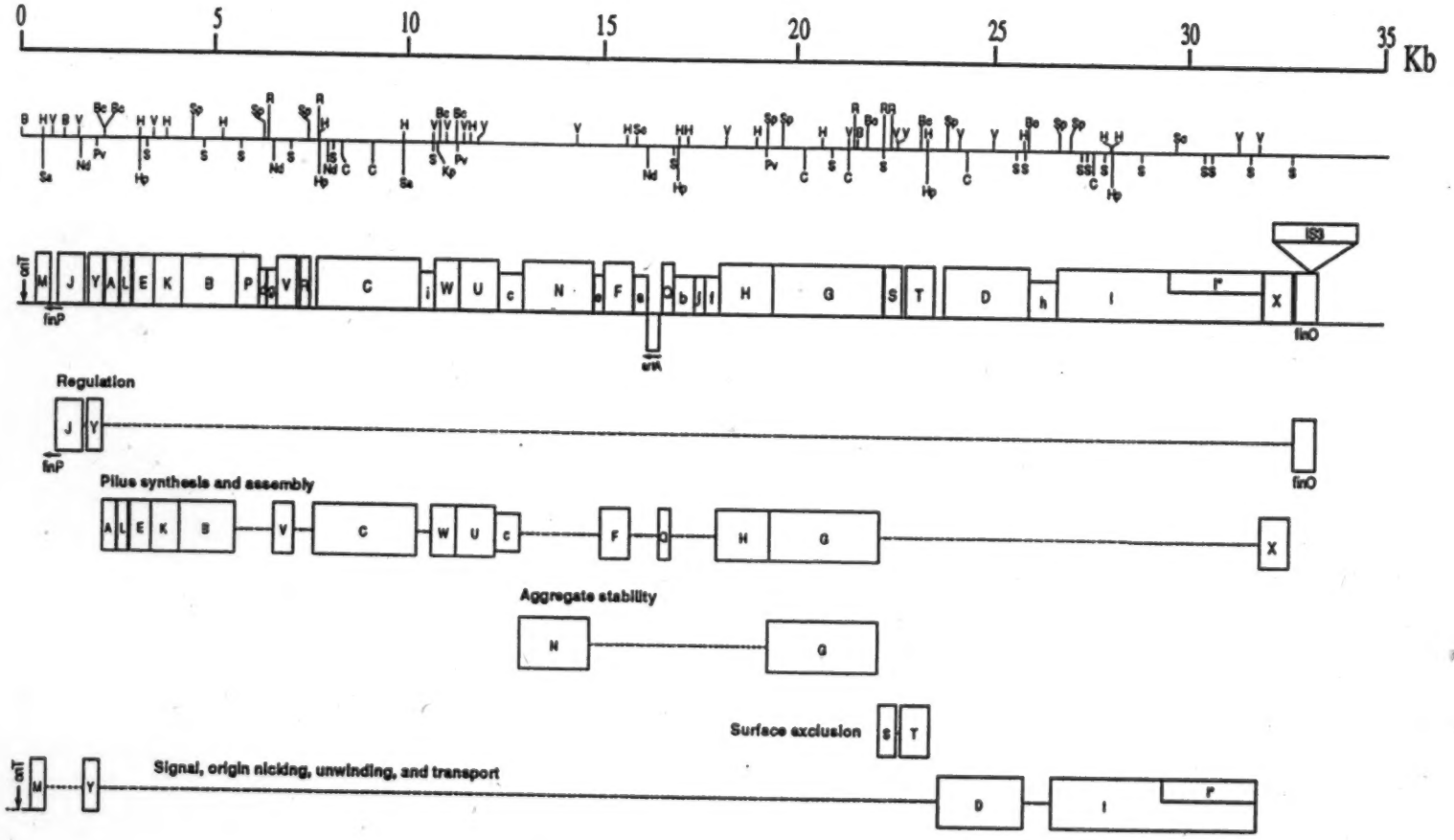
1980). (i) A specific contact between a donor (F^+) and a recipient cell (F^-) is believed to be instigated by an interaction between the tip of the pilus and a component of the recipient cell surface. (ii) Retraction of the pilus by an unknown mechanism brings the mating cells into wall-to-wall contact to form mating pairs or aggregates if more than two cells are involved in the association. (iii) These mating aggregates are stabilized in a manner that renders the aggregates more resistant to shear forces, in preparation for DNA transfer. (iv) Meanwhile, a 'mating signal' believed to be initiated by functional pair formation sets in motion the events leading to the nicking, displacement and transfer of one DNA strand into the recipient in a 5'→3' direction. Concomitant to the transfer event, replacement and complementary strand synthesis occurs in the donor and recipient cells respectively, resulting in the generation of a newly replicated plasmid in the former and establishment of the plasmid in the latter. (v) Upon the completion of this process, the mating cells actively dissagregate from each other by a mechanism not yet understood. The *tra* proteins required for each of the stages mentioned here are included in Fig. 1.2.

1.2.5 Genetic organization of the F transfer operon

Our current understanding of the nature of the transfer operon stems from the earliest complementation and mapping studies performed by Hirota and Ohtsubo using stable heterozygotes containing transfer-deficient F and R100-1 plasmids. Results from these cross-complementation studies not only identified several *tra* cistrons but also revealed the interchangibility of most of the *tra* gene products among F-like plasmids (Hirota *et al.*, 1966). A systematic mutational analysis was also carried out by Achtman and Willetts using transient heterozygotes containing two F*lac* transfer mutants (Achtman *et al.*, 1972). These two initial studies identified twelve cistrons required for conjugation and eventually led to the discovery of the operon nature of the *tra* genes and the development of a physical map.

Beginning in the 1980s, the introduction of DNA sequencing technology led to a detailed genetic and physical analysis of the *tra* operon at maximal resolution from the work

Fig. 1.2 The F transfer region. The top line indicates the length of the F transfer region in kilobases. The position of restriction sites are shown on the second line: B, *Bgl*II: Sa, *Sal*I; V, *Eco*RV; Nd, *Nde*I; Bc, *Bcl*I; H, *Hinc*II; Hp, *Hpa*I; S, *Sma*I; Sp, *Sph*I; C, *Cla*I; R, *Eco*RI; Kp, *Kpn*I; Sc, *Sca*I; Pv, *Pvu*I. The third line represents the gene and gene products of the *tra* region; *tra* genes are denoted by capital letters while *trb* genes are indicated by lowercase letters; *artA* and *finP* are transcripts in the anti *tra* orientation and *oriT* is the origin of transfer. Gene products involved in regulation of transfer, pilus synthesis, aggregate stability, surface exclusion and DNA metabolism are shown underneath the map. This map is adapted from Frost *et al.* (1994).


0
5
10
15
20
25
30
35
Kb
IS3
finP
finO
Regulation
Pilus synthesis and assembly
Aggregate stability
Surface exclusion
Signal, origin nicking, unwinding, and transport

collectively carried out by members of the Frost, Ippen-Ihler, Skurray and Willetts laboratories. The resulting map depicted in Fig. 1.2, is derived from our current state of knowledge of the *tra* operon (Frost *et al.*, 1994); it shows the organization and functional classification of the 36 open reading frames (ORFs) known or deemed likely to encode a protein product. These genes have been categorized into five functional groups namely, regulation, pilus synthesis, mating aggregate stabilization, DNA metabolism, and surface exclusion. Also included in this array are several ORFs which code for proteins of unknown function (Frost *et al.*, 1994). Products of *traJ, Y,* and *finO* and the antisense RNA, FinP, regulate the expression of the *tra* operon with TraJ being the positive regulator and FinO/FinP forming the fertility inhibition system which contribute to the repressed state of many conjugative plasmids (Frost *et al.*, 1994). The action of FinO/FinP combination inhibits the expression of *traJ*. The absence of TraJ in turn precludes transcription from the major transfer region promoter, P_Y, located at the proximal end of the operon. However, due to the insertional inactivation of *finO*, the fertility inhibition system in F is dysfunctional and as a result, the transfer of the F plasmid is derepressed. The product of *traM* is believed to be involved in transducing a signal that DNA transfer should begin. The TraI protein is an ATP-dependent helicase which unwinds and displaces a single-stranded DNA; together with TraY it is also required for the *in vivo* nicking at the origin of transfer (OriT). TraD is a large inner membrane protein believed to be involved in the transport of single-stranded DNA across the cell envelope into the recipient. The products of fifteen genes are required for the synthesis and assembly of the pilus. *traA* is the structural gene for pilin while *traQ* and *traX*, respectively encode products required for the correct processing and acetylation of the pilin precursor (Minkley *et al.*, 1976; Moore *et al.*, 1982; 1993). The remaining gene products are thought to be involved in the assembly process. The products of *traN* and *traG* are needed to stabilize mating aggregates. Surface exclusion, a process which inhibits the redundant DNA transfer between two donor cells, is brought about by the concerted actions of TraS and TraT proteins.

Transcription of a 32 kb polycistronic mRNA encoding the entire *tra* region (except *traM* and *traJ*) originates from the major P_Y promoter. The presence of independent

promoters upstream of genes located at the distal end of the operon has been well documented. These include the promoters for the surface exclusion genes, *traS* and *traT*, which allow constitutive high level expression of their protein products, as well as promoters for *traD*, *traI* and *trbF* (Jalajakumari *et al.*, 1987; Frost *et al.*, 1994). In contrast, high level synthesis of pilin is derived from a stable *traA* mRNA formed by postranscriptional processing of the long *tra* transcript at its 5' and 3' ends (Koraimann *et al.*, 1996). The *traAL* mRNA contains a clover-leaf intracistronic mRNA stabilizer at its 3' end in *traL* which might afford a longer half-life to the transcript, thus allowing high amounts of pilin synthesis through repeated translation events to generate the vast quantities of pilin (1.2×10^5 monomers/cell) for polymerization (Koraimann *et al.*, 1996; Silverman, 1987).

1.3 The Conjugative Pilus

A central feature of F plasmid-mediated conjugation is the absolute requirement for a conjugative pilus since mutations in any one of the fourteen essential genes dedicated to pilus synthesis prevent pilus formation and abolish DNA transfer (Frost *et al.*, 1994).

1.3.1 The discovery of the F pilus

During the phenomenon of conjugal pairing in *E. coli*, Lederberg (1956) demonstrated that the most notable feature of the donor bacteria was their unique ability to form 'specific unions' with recipients. Several lines of evidence suggested that this was due to the synthesis of a new surface component which was initially thought to act by lowering the net negative charge on the surface of donor cells to facilitate the formation of intimate contacts with recipient bacteria (Hayes, 1968). This property was characterized by aggregation in buffers at high pH and their low affinity for basic dyes. Subsequently, Orskov and Orskov (1960) demonstrated the existence of an antigen, termed f', that was only present in strains harbouring the F plasmid. In addition, only F' and Hfr cells but not recipient bacteria, were found to be susceptible to certain classes of spherical RNA and filamentous DNA bacteriophages (Loeb, 1960; Zinder *et al.*, 1963). Progress in understanding the basis of some of these phenotypic differences between donor and recipient strains was accomplished

by the discovery of Crawford and Gesteland (1964) that the spherical RNA phage, R17, attached along the sides of filamentous structures possessed by F' and Hfr donors. Brinton termed these structures F pili and showed that F pili are encoded by F, and that they are required for conjugation (Brinton *et al.*, 1964). Caro and Schnos (1966) later demonstrated that the filamentous DNA phage, f1, adsorbed specifically to the tip of F pili. Using an f'-specific antiserum, Ishibashi (1967) provided the experimental evidence that F pili corresponded to the f' antigen described previously by Orskov and Orskov (1960). Following the discovery of F pili, numerous other F-like plasmids including the R and Col factors have since been shown to determine the synthesis of similar pili (Datta *et al.*, 1966; Lawn, 1967; Meynell and Lawn, 1967). To distinguish these pili as a class from others found commonly on enteric bacteria, Meynell and Lawn (1967) coined the term 'sex pili' to refer to filamentous structures exclusively involved in DNA transfer.

1.3.2 Classification of sex pili of the IncF complex

All F-like conjugative plasmids specify thick flexible pili which confer the 'universal' mating phenotype to cells expressing these pili by promoting mating at equal efficiency in both liquid and on solid media (Bradley, 1980; Paranchych and Frost, 1988). Since F-like pili are morphologically indistinguishable, in general, these pili are classified principally by serology and by characteristic pilus-specific phage adsorption (Lawn and Meynell, 1970; Willetts and Maule, 1986). These studies were initiated by Lawn and Meynell (1970) who observed that antiserum specifically raised against one F-like pilus exhibited various degrees of cross-reactivity to other pili in the same group. Based on these results F-like pili were assigned into one of four serogroups, A, B, C, or D (Lawn and Meynell, 1970). Willetts and Maule (1986) further distinguished the four serogroups into four pilus types, I, II, III, and IV on the basis of the degree of sensitivity they conferred to pilus-specific RNA and DNA phages. The number of pilus types were further expanded to six (I, IIA, IIB, III, IV and V) using a more stringent classification scheme (Frost *et al.*, 1985). Under these criteria, the F plasmid specifies pili of Serotype A and Type I, whereas R100-1 pili belong to Serotype D and Type IV while the pili of ColB2 are of Serotype C and Type IIA (Frost *et al.*, 1985). F pili attach both DNA and

RNA phages efficiently, thus cells harbouring the F plasmid are fully susceptible to these phages. Although R100-1 pili attach DNA phages well, they adsorb RNA phages with much reduced efficiency; however, R100-1 carrying cells exhibit resistance to both phages. Although ColB2 pili adsorb both phages, cells carrying this plasmid are susceptible only to RNA phage infection but not to DNA phages. Presumably, F-like pili differ structurally, albeit very subtly from each other, and these differences might be the basis for the variation in RNA and DNA phage infection (Tomoeda *et al.*, 1975). Nevertheless, the overall similarity of F-like pili must be emphasized as cells carrying two different F-like plasmids can assemble pili containing a mixture of two different pilin subunits (Lawn *et al.*, 1971).

1.3.3 Structural and biochemical properties of F pili

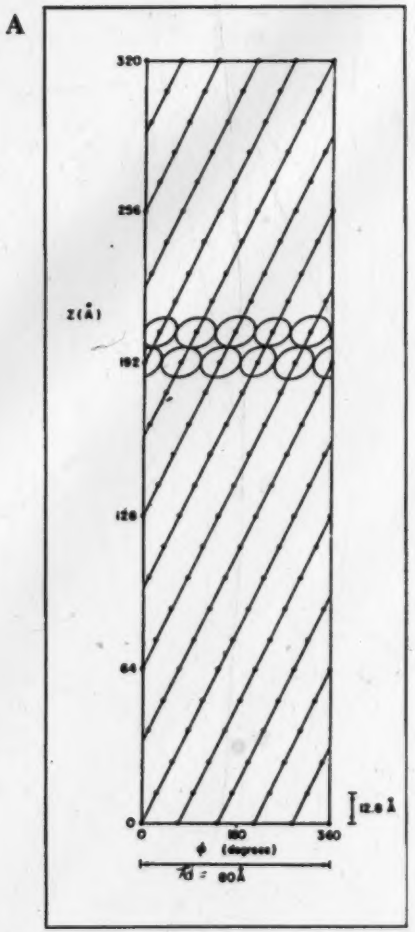
In the electron microscope, all thick, flexible F-like pili appear as long filamentous rods of approximately 1-2 μm long with a characteristic knob at the base of free pili (Meynell, 1978). A dark axial line representing an inner channel can usually be discerned in negatively stained pili preparations (Frost, 1993). Unlike common pili which are present in vast numbers, an F^+ cell expresses on average 1-2 pili per cell (Paranchych and Frost, 1988).

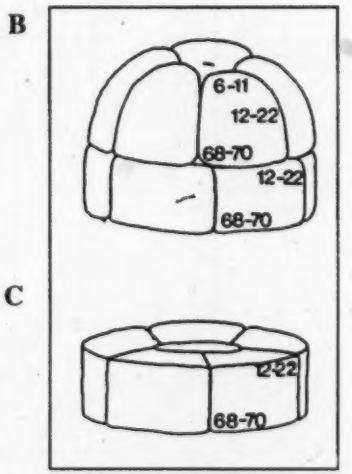
The physical and chemical nature of F pili was first characterized by Brinton (1971) who demonstrated that the F pilus is an assembly of a single type of subunit, F pilin. The pilin subunit was described as a phosphoglycoprotein with a molecular weight of 11 800 containing two molecules of phosphate and one molecule of D-glucose in covalent association (Brinton, 1971). Using electron microscopy, Brinton also postulated the fine structure of the F pilus to be a polymer composed of two parallel strands of protein, each consisting of an assembly of pilin monomers. By heating a purified F pili preparation to 55°C at pH 4.5 in sodium pyrophosphate, Brinton found that the rods in the pilus filament were converted into fibres capable of self-reassembly *in vitro* upon removal of the denaturant. However, further treatment of F pili at 55°C at pH 2.0 in sodium pyrophosphate, irreversibly altered the fibres to vesicles reminiscent of the knobs at the base of free pili. This led Brinton to suggest that F pilin can exist in several morphologically distinct forms and that the terminal knobs are vesicles containing a different form of pilin (Brinton, 1971).

In stark contrast to Brinton's findings, a revised helical model for the F pilus was proposed from optical and X-ray fibre diffraction analyses using the correct molecular weight of F pilin of 7 200, by Folkhard *et al.* (1979) and Marvin and Folkhard (1986). As illustrated in Fig. 1.3, this model envisions a helical arrangement of pilin monomers to form a hollow cylinder measuring 8 nm in outer diameter with a 2 nm axial hole in the centre. The basic helix contains 25 pilin subunits in two turns with a pitch of 16 nm. The unit rise is 1.28 nm and the crystallographic repeat is 32 nm. The subunits are related by five-fold symmetry around the pilus axis. Hence, for simplicity, pili can be visualized as consisting of doughnut-shaped discs composed of five segments each corresponding to a pilin subunit; the discs are stacked such that each is rotated 28.8° with respect to the disc below (Frost, 1993; Paiva *et al.*, 1992). Alternatively, the pilus can be viewed as a polymer composed of five strands of assembled pilin wound together in a helical fashion (Firth *et al.*, 1996).

Analysis of the nature of carbohydrates and phosphates associated with F pilin has been the subject of several studies in an attempt to gain an insight into the mechanism of F pilin synthesis and assembly (Brinton, 1971; Date *et al.*, 1977; Armstrong *et al.*, 1981). Armstrong *et al.* (1981) found that each F pilin subunit contains the equivalent of two to three hexose units as well as two phosphate residues. However, further attempts to visualize phosphates in intact pili using electron spectroscopic imaging revealed that there was no evidence of phosphate either in the pilus filament or at the terminal knobs (Frost and Bazett-Jones, 1991). In addition, recent studies to reconfirm the molecular weight of pilin of the IncFV plasmid, pED208, using mass spectroscopy identified only a single type of subunit of 6 800 in pED208 pili corresponding to the size of acetylated pilin determined from genetic analysis (Frost, unpublished data; Finlay *et al.*, 1986). Collectively, these observations support the notion that previous reports of phosphate and carbohydrate in pili are illusory and that they could have been derived from traces of contaminating membrane material that is often seen in pili preparations (Meynell, 1978).

Fig. 1.3 A representation of the physical parameters of F pili. A. The F pilus is represented as a five-start helix with a rise per subunit of 1.28 nm and a helix symmetry of 25 subunits in two turns of the helix with a crystallographic repeat of 32 nm. A sketch of a single layer of five subunits in the pilus (C) as well as a representation of a possibly different packing of the subunits at the tip are also presented (B). The numbers 6-11 represent the amino acids in mature pilin thought to be important for f1 phage attachment (Section 1.4.5); numbers 12-22 and 68-70 represent the sites of attachment for R17 and Qβ phages, respectively (Section 1.4.4). Figures 3A, B and C are adapted from Frost, 1993. Fig. 3D is an electron micrograph of an F pilus laterally coated with spherical R17 phages and with a filamentous f1 phage attached to the tip (total magnification is 83 750x).


A
320
256
192
128
64
0
z(Å)
0
180
360
φ (degrees)
12.8 Å
2πR = 80Å


B
6-11
12-22
68-70
12-22
68-70
C
12-22
68-70

D

1.3.4 Factors affecting the kinetics of F pilus assembly and disassembly

Since the early 1960's, a vast amount of literature has been compiled on studies performed to understand the nature of assembly and disassembly of the F pilus. These studies which mainly focused on the effects of environmental and host-encoded factors, mechanical force, temperature, metabolic inhibitors, chaotrophic agents, pilus-specific antiserum and bacteriophages on the appearance and disappearance of the pilus, have collectively contributed significantly to our current understanding of the nature of the pilus.

Production of pili in a culture growing aerobically in rich medium parallels closely to the growth phase, such that the maximum number of pili (1-2 per cell) appears in the late exponential or early stationary phase (Tomoeda, 1975). As the culture reaches stationary phase, pili disappear from the cell surface giving rise to F phenocopies which are F^+ cells with very poor donor ability (Ippen and Valentine, 1965; Jacob and Wollman, 1961). Under anaerobic conditions however, for reasons unknown, donor cells produce pili that are longer than normal and three times as many in number (Curtiss, 1969; Tomoeda, 1975). Although intracellular levels of cyclic AMP (cAMP) have also been reported to influence transfer-related properties of F-like plasmids, this effect is not evident for F pili (Harwood and Meynell, 1975). However, Kumar and Srivastava (1983) observed that *cya* and *crp* mutant hosts harbouring the F plasmid exhibited a reduction in transfer-related properties and concluded that F *tra* expression is dependent on cAMP and its receptor protein, CRP. Mutations affecting the host *cpxA* and *sfrA* genes whose products represent transducer and effector homologues respectively of the two-component regulator protein families, appear to play regulatory roles in the expression of F transfer functions (McEwen and Silverman, 1980; Firth *et al.*, 1996). As such, these factors may influence pilus outgrowth indirectly by modulating *tra* gene expression.

Novotny *et al.* (1969) determined that F pili removed from the cell surface by blending are rapidly replaced by newly synthesized pili. The effect of cultivation temperature on pilus outgrowth was demonstrated by Novotny and Lavin (1971), who showed that the maximum number of F pili occur between 37°C-42°C; however, at 25°C pilus outgrowth ceases and at 20°C or 50°C (Novotny and Fives-Taylor, 1978) approximately 50%

of pili disappear from the cell surface. Quick chilling to 0°C however, promotes neither pilus outgrowth nor its disappearance. Collectively, these studies suggest that the synthesis, maintenance and detachment of F pili require metabolic activity (Tomoeda, 1975). Novotny proceeded to show that inhibitors of protein and DNA synthesis did not have an immediate effect on pilus synthesis; however, Fives-Taylor and Novotny (1974) found an interesting correlation between the incorporation of 5-bromouracil into DNA and the increase in the length of F pili. These observations led the authors to conclude that pili are synthesized from a pool of pre-existing pilin subunits and that DNA may somehow be associated with F pili.

Treatment of piliated F^+ cells with energy poisons such as sodium cyanide, dinitrophenol, sodium azide and sodium arsenate resulted in the rapid disappearance of cell associated pili without concomitant increase in the number of pili in the medium; removal of these agents, however, restored pilus production (Novotny and Fives-Taylor 1974; O'Callaghan *et al.*, 1973). This evidence indicates that F pilus synthesis and maintenance are energy-requiring and reversible processes while retraction or disappearance of F pili from the cell surface is energy-independent.

The addition of pilus-specific antiserum had an unusual stimulating effect on the synthesis of thin I-like conjugative pili as observed by the presence of over fifty pili per cell on cells treated with a pilus-specific antiserum in contrast to one or two on untreated cells (Lawn and Meynell, 1972). Similar results were also obtained when piliated *Pseudomonas aeruginosa* cells were allowed to adsorb RNA phages (Bradley, 1972). Although conclusive evidence showing an increase in the number of F pili has not been obtained, these observations offer interesting perspectives on the mechanism of pilus assembly. The increase in pilus number upon attachment of pilus-specific antiserum or RNA phages could be explained if pilus assembly and disassembly depended on an equilibrium governing the continuous extrusion and retraction of pili. Thus, binding of either antiserum or RNA phages may prevent retraction which in turn increases the number of pili in the extruded state (Tomoeda, 1975). Alternatively, attachment of antiserum or RNA phages may bring about extrusion by upsetting the normal equilibrium between free pilin subunits and polymerized pilin (Tomoeda, 1975).

Physically, pili are rather insoluble structures which exhibit a tendency to form large aggregates by themselves or clumps with cells (Tomoeda, 1975). F pili are also resilient to treatments with chaotrophic agents such as urea, guanidine hydrochloride, Brij 58, deoxycholic acid, heat or alkali (Frost, 1993). However, they disaggregate in the presence of sodium dodecyl sulphate (SDS) or sarkosyl and form vesicle-like structures when subjected to pH 1.0 (Frost, 1993). Treatment with HCl, pyrophosphate or Triton X-100 causes the unwinding of the F pilus along its longitudinal axes into thinner filaments while sonication causes disruption of the pilus into discs presumably equivalent to one layer of five subunits (Frost, 1993).

1.4 Synthesis of F Pilin

Pilin which is the major structural protein of F pili has been extensively studied; its synthesis, topology and functional domains are well understood. In the following sections, an analysis of the pilin polypeptide beginning from its synthesis to its assembly is presented.

1.4.1 Identification and characterization of the pilin gene

The identification of the F pilin gene was preceded by Willetts' initial observation that complementation of the F*lac traA1* mutation with the R100-1 plasmid, resulted in a transfer-proficient phenotype with severely reduced ability to plate the RNA phage, R17. This suggested that *traA* may be the structural gene for pilin and that the differences in R17 sensitivity might reflect the differences in phage attachment brought about by variation in the two pilin proteins (Willetts, 1971). Subsequently, Minkley *et al.* (1976) confirmed this notion by analyzing the presence of radioactively iodinated tyrosine residues in pili produced by the F*lac traA1* amber mutant in a tyrosine amber suppressor strain. Determination of the nucleotide sequence of F *traA* (Frost *et al.*, 1984) revealed an open reading frame of 121 codons encoding a 12.8 kDa propilin precursor. However, the pilin subunit purified from F pili was determined to be a 7.2 kDa polypeptide corresponding to the last 70 amino acids in TraA with an acetylated N-terminus (Frost *et al.*, 1984). Thus, the mature form of pilin was

the product arising after the removal of an unusually long 51 amino acid leader peptide from propilin followed by subsequent N-terminal acetylation (Frost *et al.*, 1984).

1.4.2 Roles of TraQ and TraX in F pilin maturation

The conversion of propilin precursor into mature pilin requires two additional steps carried out by two separate *tra* proteins. The processing and N-terminal acetylation steps in propilin maturation depend on the expression of *traQ* and *traX*, respectively (Moore *et al.*, 1982; Moore *et al.*, 1993).

The *traQ* gene was first identified as essential for pilin synthesis because F plasmid derivatives lacking this region of the *tra* operon were unable to accumulate pilin in the inner membrane (Moore *et al.*, 1982). Analysis of the *traQ* gene product revealed a 10.9 kDa inner membrane protein (Wu and Ippen-Ihler, 1989) whose function is required for the efficient processing of the 12.8 kDa pilin precursor to generate the 7 kDa intermediate pilin species prior to acetylation. In the absence of TraQ, misfolding of propilin is thought to interfere with its membrane translocation which subsequently leads to its rapid degradation. Consequently, only a small percentage of propilin is processed (Maneewannakul *et al.*, 1993).

Recent evidence indicates that TraQ provides an efficient *sec*-independent pathway for the rapid insertion of propilin polypeptide into the inner membrane upon which the signal sequence is removed in a single cleavage step by LepB signal peptidase (Majdalani and Ippen-Ihler, 1996). This process is dependent on the proton motif force (pmf) as an energy source (Majdalani and Ippen-Ihler, 1996). In contradiction to previous notions that TraQ might be required to cleave the first 25-30 amino acids from the unusually long 51 aa signal sequence in order to expose the recognition sequence for LepB, TraQ was found not to possess any endopeptidase activity (Majdalani *et al.*, 1996). Moreover, gene fusions in which the *traA* signal sequence was fused to either *phoA* or β-lactamase genes resulted in efficient expression of active alkaline phophatase and β-lactamase in both F^+ and F^- cells, indicating that it is the sequences within the mature form of propilin and not the signal sequence that dictate the TraQ-dependence of propilin maturation (Paiva and Silverman, 1996; Majdalani *et al.*, 1996). Since TraQ is not directly involved in propilin leader cleavage but is necessary for

propilin translocation, Majdalani *et al.* (1996) proposed that TraQ may either act as a chaperone to prevent propilin misfolding and maintain it in a form competent for membrane insertion, or alternatively, it may form a propilin-specific membrane channel which promotes rapid insertion of the precursor into the inner membrane. However, since *sec*-independent translocation presumably occurs through the lipid-portion of the cytoplasmic membrane rather than through a protein channel, the former is a more conceivable role for TraQ (Pugsley, 1993).

The realization that an additional *tra* product is required for the synthesis of mature F pilin stemmed from the observations that pilin polypeptides expressed from TraA$^+$ TraQ$^+$ constructs migrated at a faster rate on SDS-polyacrylamide gels than pilin from purified pili (Laine *et al.*, 1985). Furthermore, these pilin polypeptides failed to react with the F pilus-specific antiserum which predominantly consists of antibodies targeted against the immunodominant acetylated N-terminus of F pilin (Laine *et al.*, 1985; Finlay *et al.*, 1985). Subsequently, the *traX* cistron was mapped to the distal end of the *tra* operon between *traI* and *finO*; nucleotide sequence analysis of this locus predicted an open reading frame encoding a 248 aa, 27.5 kDa polytopic inner membrane polypeptide (Cram *et al.*, 1991; Moore *et al.*, 1993). *In vivo* and *in vitro* analyses have detected two *traX* products, TraX1 and TraX2 of 24 kDa and 22 kDa, respectively, associated with the inner membrane (Maneewannakul *et al.*, 1995). These significantly smaller products are assumed to originate from translational reinitiation events at start codons located downstream of the first methionine (Maneewannakul *et al.*, 1995). Interestingly, translation initiation at the first methionine enhances and thus regulates subsequent translation reinitiation events. Residues 29-225 of TraX are essential for acetylation, however, the significance for the presence of two products is not understood (Maneewannakul *et al.*, 1995). Surprisingly, N-terminal acetylation of pilin is not essential for conjugation or for pilus-specific phage infection since an F plasmid derivative carrying an insertion mutation in the *traX* gene expresses normal numbers of pili composed of unacetylated pilin subunits and exhibits wild type levels of DNA transfer and phage susceptibility (Maneewannakul *et al.*, 1995). Although pilus assembly seems normal, there is evidence that the properties of pili produced by an F *traX* mutant

differ somewhat from those of wild type F pili since cells carrying the F *traX* mutant exhibit a high degree of clumping in liquid culture suggesting that pili containing unacetylated subunits are more adhesive (Maneewannakul *et al.*, 1995). In addition, pili synthesized by pTG801, a multicopy plasmid which expresses all the F pilus synthesis genes except *traX*, under the control of the *lac* promoter, also showed unusual properties (Grossman and Silverman, 1989; Grossman *et al.*, 1990). Immunogold electron microscopy studies using a monoclonal antibody, JEL92, specific for a region near the pilin N-terminus (Fig. 1.5; Frost *et al.*, 1986), indicated that unlike wild type pili the N-terminal region of pilin was more exposed along the length of pTG801 pili (Grossman *et al.*, 1990). In addition to the differences in immunoreactivity, these pili also exhibited poor ability to adsorb R17 phages as well as an unusually adhesive property as shown by their propensity to form lateral aggregates (Grossman *et al.*, 1990). Whether these differences reflect an overall perturbation in the packing arrangements of pilin subunits in the pilus filament, or a more localized difference in subunit conformation confined to the amino terminus, is not clear (Silverman, 1997). Despite the dispensable nature of N-terminal acetylation of F pilin for pilus biogenesis and function, and the relatively rare nature of this type of modification in prokaryotes, this might be a common phenomenon shared by all F-like systems since the presence of N-acetylated pilin or a *traX* homologue has been documented in several F-like plasmids (Maneewannakul *et al.*, 1995; Firth and Skurray, 1995; Frost *et al.*, 1985; Yoshioka *et al.*, 1990; Cram *et al.*, 1991).

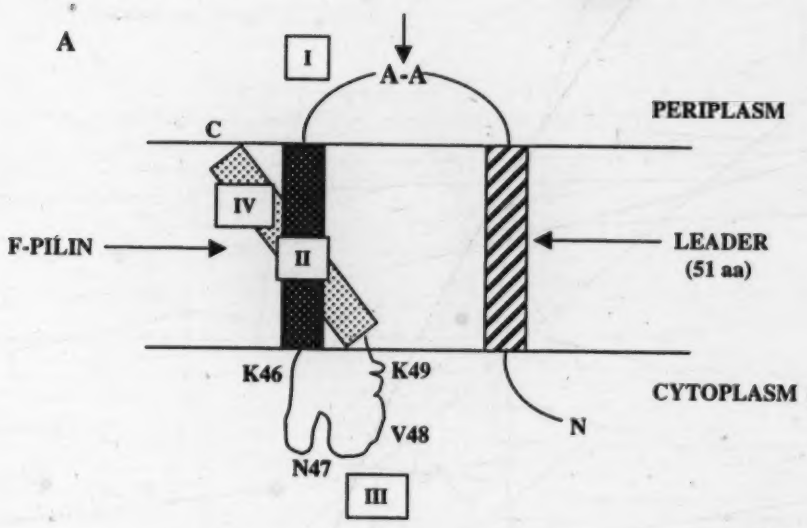
Apart from signal sequence processing and N-terminal acetylation, an additional form of modification on a small proportion of F pilin subunits has been suggested (Laine *et al.*, 1985; Moore *et al.*, 1993). Recently, it was demonstrated that a minor subset of pilin in free pili as well as in the inner membrane pool bear an uncharacterized form of modification which retarded their migration on SDS-polyacrylamide gels (Moore *et al.*, 1993). These 8 kDa subunits contain an acetylated N-terminus and exhibit considerable stability *in vivo* (Maneewannakul *et al.*, 1993). This species is thought to arise from a modification event at multiple sites within pilin carried out by an unknown host enzyme; this event appears to occur subsequent to signal sequence cleavage but prior to acetylation (Maneewannakul *et al.*,

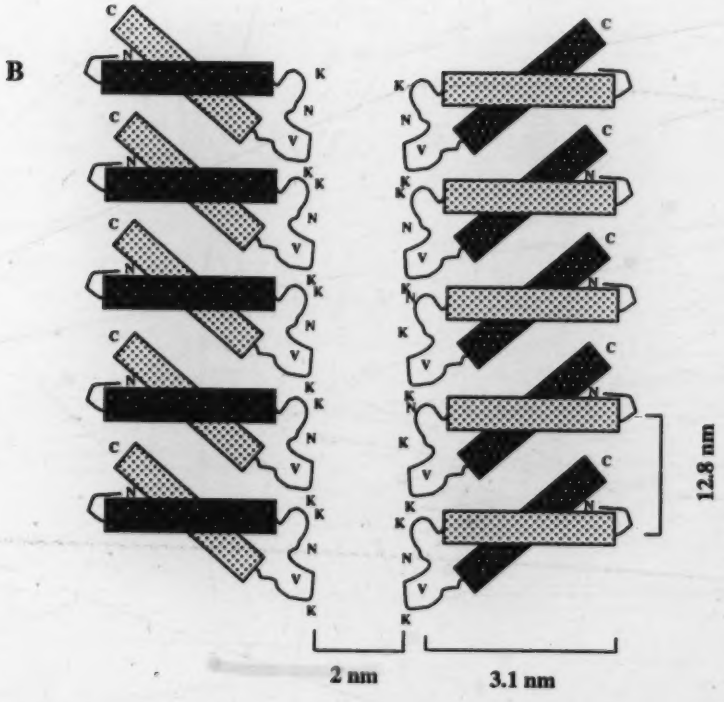
1993). Pulse chase experiments indicated that in a wild type F' strain, the amount of the 8 kDa pilin in the inner membrane stabilized over time while in a *traX* mutant an increase in its quantity was observed, suggesting that N-terminal acetylation inhibits the modification process (Maneewannakul *et al.*, 1993). As further support to this notion, Maneewannakul *et al.*(1993) found that purified wild type F pili contained only a minor proportion of the modified form of pilin as compared to pili from the F *traX* mutant which had a mixture of the modified and unmodified forms in approximately equal ratio. However, contradictory findings from mass spectroscopic analysis which identified only a single type of pilin monomer of 6.8 kDa in pED208 pili, and the absence of phosphate or glucose in covalent association with pilin (Section 1.3.3), question the significance of the presence of the 8 kDa pilin subunit. The possibility that either lipopolysaccharide or phospholipid contamination of pilin gives rise to the 8 kDa subunit cannot be ruled out.

1.4.3 Topology of the F pilin subunit

Under normal growth conditions approximately 85% ($1x10^5$) of pilin subunits in an F' cell seem to be associated with the inner membrane which is believed to be a reservoir for pilin during pilus assembly and disassembly (Moore *et al.*, 1981a and 1981b; Silverman, 1987; Sowa *et al.*, 1983). Hydropathy analysis identified four domains in mature pilin and structural studies revealed its topology in the inner membrane (Paiva *et al.*, 1992). Immunogold electron microscopy of spheroplasts using the acetylated N-terminal-specific monoclonal antibody (mAb), JEL93 (Fig. 1.5; Frost *et al.*, 1986), revealed that the N-terminus of F pilin resided in the periplasm (Paiva *et al.*, 1992). Using *traA-phoA* gene fusions, the topology of F pilin in the inner membrane as shown in Fig. 1.4A was derived. The U-shaped F pilin is oriented such that its N- and C-terminal residues are located in the periplasm. Two hydrophobic segments spanning the inner membrane are connected by a tetrapeptide loop on the cytoplasmic side (Paiva *et al.*, 1992; Paiva and Silverman, 1996). Based on the physical parameters of F pili proposed by Marvin and Folkhard (1986), and the 70% α-helical content of F pilin determined by circular dichroism studies (Armstrong *et al.*, 1980; Date *et al.*,

Fig. 1.4 Topology of F pilin subunit in the inner membrane (A) and a proposed structure of F pilin in the filament (B). The *traA* leader sequence is proteolytically cleaved at the two alanine residues (arrowhead); the orientation of the four functional domains of pilin in the inner membrane described in the text are shown in A. N and C indicate the amino and carboxy termini, respectively. The spatial arrangement of the four functional domains of pilin shown in B, indicate the positioning of the tetrapeptide, KNVK, in the lumen of the pilus and the C-terminus exposed laterally. The N-terminus is shown in close association with the C-terminus but buried within the filament. The physical parameters of the pilus as described in Section 1.3.3 are also shown. These figures were adapted from Paiva *et al.* (1992) with a slight modification to include the N-terminus in B.


A
I
A-A
PERIPLASM
C
IV
F-PILIN
II
LEADER
(51 aa)
K46
K49
CYTOPLASM
V48
N
N47
III


B
12.8 nm
2 nm
3.1 nm

1977), Paiva *et al.* (1992) devised a model to illustrate the spatial and domain organizations of F pilin in the pilus filament. As shown in Fig. 1.4B, this model contains two antiparallel α-helices comprising the two transmembrane segments corresponding to domains II and IV of pilin, forming a hydrophobic 'girdle' (Folkhard *et al.*, 1979) surrounding the lumen. The arrangement is such that the two domains extend from the central axis towards the periphery of the filament at a defined angle. A critical feature of the proposed structure is the turn joining the two α-helical domains which contains the basic tetrapeptide, KNVK, in domain III positioned in the hydrophilic lumen implying a possible interaction between domain III and the acidic backbone of DNA (Silverman, 1997). In addition, the model proposes that domain I of F pilin is oriented away from the filament axis towards the aqueous milieu and possibly interacts with the C-terminus (Fig. 4B). However, immunogold electron microscopy studies of F pili using JEL92 (specific for residues surrounding methionine 9; Fig. 1.5) and JEL93 (specific for the acetylated N-terminus; Fig. 1.5) monoclonal antibodies, revealed that the region extending from the acetylated alanine to methionine 9 in domain I which defines the epitopes for these antibodies, is neither exposed on the pilus periphery nor at the pilus tip (Frost *et al.*, 1986). Interestingly, mAb JEL92 coats the sides of pTG801 pili indicating a deviation from the normal pattern of subunit assembly in these pili in the absence of pilin N-terminal acetylation (Grossman *et al.*, 1990).

1.4.4 Structural and functional dissection of the F pilin subunit

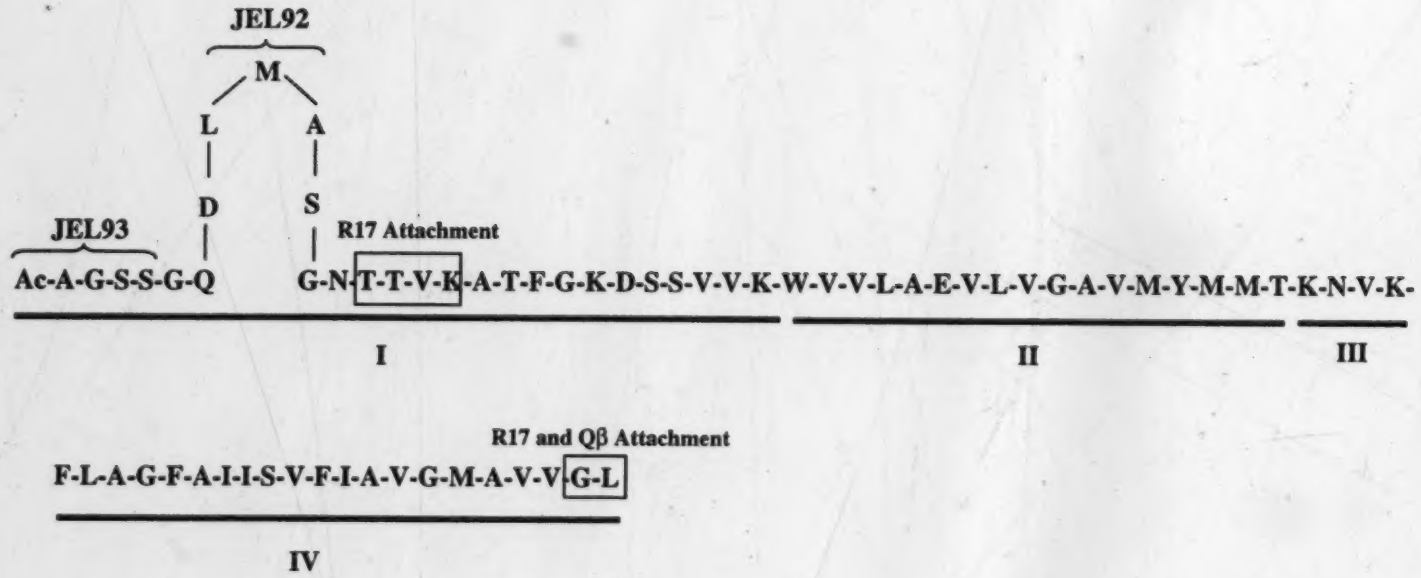
The development of pilus-specific polyclonal antisera and monoclonal antibodies (mAb) JEL92 and JEL93, as well as the availability of pilus-specific phages have greatly facilitated the functional and structural assignment of specific F pilin residues.

The epitope on pilin specifically recognized by the mAb JEL92 is thought to contain a β-turn comprising seven residues centred at M_9 in the mature pilin, while mAb JEL93 recognizes the tetrapeptide, Ac-A_1-G_2-S_3-S_4, at the N-terminus with the acetyl group being an important feature for the antigenicity of this peptide (Fig. 1.5; Frost *et al.*, 1986). Immunogold electron microscopy studies revealed that both JEL92 and JEL93 bind to the knobs at the base of F pili with high affinity, but neither bind to the shaft or the tip indicating

that the regions defining these epitopes are not exposed on the surface of the pilus filament, but are accessible in the unassembled pilin in the knobs. These observations confirm earlier notions that the configuration of pilin in the knobs is different than in the filament (Frost *et al.*, 1986; Frost and Bazett-Jones, 1991; Brinton, 1971)). The observation that pili sheared by sonication contain a higher number of binding sites for mAb JEL92 than intact pili (Frost *et al.*, 1986), suggests that residues 7-12 of pilin (Fig. 1.5) may be involved in subunit-subunit interaction and that this region becomes exposed in broken pili. Over 80% of antibodies in a polyclonal F pilus antiserum are specific for the immuno-dominant acetylated N-terminus while the remaining 20% constitute antibodies targeted against a minor epitope at the C-terminus of pilin which is exposed on the pilus shaft (Worobec *et al.*, 1986).

Missense mutations in pilin which affect pilus synthesis or the attachment of R17 and the immunologically distinct RNA phage, Qβ (Frost, 1993), to the sides of F pili have helped further delineate functionally important residues in pilin (Willetts *et al.*, 1980; Frost and Paranchych, 1988). Missense mutations resulting in a serine substitution at either Gly_9 or Pro_{13} of the leader sequence (Fig. 1.5), abolishes pilus synthesis suggesting that this region is essential for pilin maturation (Frost and Paranchych, 1988). Pili expressed by F plasmid variants containing a Glu_{17} in place of Lys in mature pilin (Fig. 1.5), fail to attach R17 but attach Qβ; however, pili carrying an Asp substitution at the penultimate Gly attach R17 poorly but do not attach Qβ (Frost and Paranchych, 1988). In agreement with the results obtained from immunological studies, these observations confirm that the C-terminus of pilin is exposed on the sides of pili and is probably in close alignment with a region surrounding residues Thr_{14}-Lys_{17} which are important for R17 attachment whereas the residues located at the C-terminus are important for Qβ attachment. Both Lys_{22} and Trp_{29} appear to be crucial for pilus assembly as substitutions of either of these residues result in apiliate phenotypes (Frost and Paranchych, 1988). Replacing Lys_{47} which is believed to be positioned in the lumen of the pilus and perhaps important in mediating interactions with DNA, with a Thr also abolishes pilus synthesis (Anthony and Frost, unpublished results). Rondot *et al.* (1998) found that replacing the terminal Leu to a Ser also abolished pilus assembly but interestingly

Fig. 1.5 The sequence of mature F pilin. The amino acid sequence of mature F pilin with the acetylated (Ac) N-terminus is depicted (Frost *et al.*, 1984). Residues defining the epitopes for the monoclonal antibodies, JEL92 and JEL93, and the predicted β-turn centred around M_9 are shown as well as the residues important for attachment of R17 and Qβ phages (Frost *et al.*, 1986; Frost and Paranchych, 1988). The underlined segments correspond to the domains shown in Fig. 1.4A.


JEL92
M
L
A
D
S
JEL93
R17 Attachment
Ac-A-G-S-S-G-Q
G-N-T-T-V-K-A-T-F-G-K-D-S-S-V-V-K-W-V-V-L-A-E-V-L-V-G-A-V-M-Y-M-M-T-K-N-V-K-
I
II
III
R17 and Qβ Attachment
F-L-A-G-F-A-I-I-S-V-F-I-A-V-G-M-A-V-V-G-L
IV

an F pilin derivative carrying a 17 aa extension at the C-terminus was correctly processed and assembled into fully functional pili with the peptide displayed on the lateral surface.

1.4.5 The structural basis for the phenotypic variation of F-like pili

The basis underlying the variation in serology and pilus-specific phage susceptibility among F-like pili has been attributed to the differences in the primary sequence of their pilin proteins (Lawn and Meynell, 1970; Willetts and Maule, 1986). The DNA sequence of the *traA* alleles from several F-like plasmids including R100-1, ColB2, R1-19 (IncFIII) as well as the more distantly related IncFV plasmid, pED208, has been determined (Finlay *et al.*, 1984; Frost *et al.*, 1985; Finlay *et al.*, 1986). A comparison of these pilin sequences is presented in Fig. 1.6. The overall conservation of the pilin structure is evident among all five proteins including the 51-55 aa leader sequence as well as the N-terminal acetylation in ColB2 and pED208 pilin. With the exception of pED208, the four pilin proteins show a high degree of sequence identity with the only conspicuous changes clustering at the N-terminus, where the F sequence, Acetyl-Ala-Gly-Ser-Ser-Gly-Gln-Asp, is replaced by the shorter sequences Acetyl-Ala-Gln-Gly-Gln-Asp in ColB2 and R1-19, and by Ala-Gly-Lys-Gly-Asp in R100-1 although N-terminal acetylation of R1-19 and R100-1 pilin proteins still needs to be proven experimentally (Fig. 1.6). In addition, R1-19 pilin contains a Thr substitution and an additional Lys at the C-terminus whereas the R100-1 pilin sequence contains six single defined substitutions (Fig. 1.6). Despite the small differences, the immunodominant N-terminus forms the basis for the differences in serology among F-like pilin since the majority of the antibodies present in pilus-specific polyclonal antisera are directed against the acetylated N-terminus (Worobec *et al.*, 1986). The observed cross-reactivity of pilus-specific antisera among F-like plasmids, however, is mainly due to the sharing of the laterally exposed minor epitope at the C-terminus and the degree of their serological relatedness has been shown to correlate well with the extent of similarity of the C-terminal sequences (Meynell, 1978; Frost *et al.*, 1985). However, subtle differences in the cross-reactivity of pilus antisera have also been detected. For instance, anti-F pilus antibodies can bind to R1-19 pili but with

Fig. 1.6 Sequence comparisons of pilin from F-like plasmids. The amino acid sequences of F propilin leader peptide (A) and the F pilin subunit (B) are shown. Below the sequence, only the residues that differ in the products encoded by the *traA* alleles of F-like plasmids are indicated (Frost *et al.*, 1985). Ac* indicates an acetyl group. Amino acid substitutions resulting from *traA* missense mutations discussed in Section 1.4.4 are indicated above the F sequence (Frost and Paranchych, 1988).

A. Propilin Leader

```
                 S   S
F        MNAVLSVQGASAPVKKKSF FSKFT R LNMLR LARAVIPAAVLMMFFPQLAMA 51
ColB2    -------------------- ----- - ----- ---------------------- 51
R1-19    --T----------------- ----- - ----- ---------------------- 51
R100-1   --T-----------E---- ----- - ----- -V-----V-----L--E---- 51
pED208   --LSFAKG-LP----NRAWQYCQMAW-GVTSKKA-S-LAALSPL-LLGVG-M-S- 51
```

B. Pilin Subunit

```
                          E   N       I                A                        DS
F        Ac*AGSSGQDLMASGNTTVKATFGKDSSVVKWVVLAEVLVGAVMYMMTKNVKFLAGFAIISVFIAVGMAVVGL 70
ColB2      Ac*AQ-------------------------------------------------------------------- 68
R1-19        AQ-------------------------------------------------------------T-----K 69
R100-1       AGKG----K--D---------F-I-------------------------V-------------S----  68
pED208       Ac*T--L-G-KDD------A--F-MMCIII--LI--VA--IR---LLI-L-LVVVI--TT--LTFIK   64
```

a much lower affinity than anti-ColB2 pilus antibodies even though the C-terminal sequences of F and ColB2 pili are identical (Worobec *et al.*, 1986). The presence of a lysine at the C-terminus of R1-19 pilin which otherwise resembles ColB2 pilin, is thought to contribute to the lowered reactivity with F-pilus antibodies (Worobec *et al.*, 1986). This also suggests that there might be subtle differences in the conformations of the pilin subunits among F, ColB2 and R1-19 pili since the terminal lysine seems to block the attachment of anti-F pilus antibodies much more effectively than anti-ColB2 pilus antibodies (Worobec *et al.*, 1986). The conformational differences have been attributed to the variation in the size of the pilin proteins since F pilin is 70 aa long while ColB2 and R1-19 pilin proteins contain 68 and 69 residues, respectively (Fig. 1.6; Worobec *et al.*, 1986). In this respect, a difference in R100-1 pilus conformation is also expected since its pilin contains 68 residues (Fig. 1.6). The mAb JEL92 reacts strongly with ColB2 and R1-19 pilin both of which possess the epitope, but not with R100-1 pilin presumably due to the presence of Lys_{11} and Asp_{14} which may alter the antigenicity of this region of pilin (Frost *et al.*, 1985). The pED208 pili, however, has diverged extensively from the F lineage as shown by the lack of homology in its pilin sequence (Fig. 1.6; Frost *et al.*, 1985).

R17 phages attach with high affinity to the shaft of both F and ColB2 pili since residues 14-17 and those at the C-terminus implicated in phage attachment, are highly conserved. Thus, cells harbouring these plasmids are fully susceptible to R17 infection (Meynell, 1978; Finlay *et al.*, 1984). Reduced affinity of R17 binding to R100-1 pili presumably due to the presence of Asp_{14}, and the lack of adsorption to R1-19 pili due to the presence of Lys_{69}, render the cells carrying these plasmids resistant to R17 infection (Fig. 1.5; Frost and Paranchych, 1988; Nishimura *et al.*, 1967; Meynell, 1978).

The filamentous DNA phages, f1, M13 or fd, which attach to the tip of the pilus, infect cells carrying the F-like plasmids to varying degrees (Lawn *et al.*, 1967). While cells carrying the F plasmid are fully sensitive, cells expressing either ColB2 or R100-1 pili confer 20% and 2% respectively, of the plating efficiency of F (Finlay *et al.*, 1984; Willetts and Maule, 1986). Variation in phage sensitivity does not appear to reflect differences in pilus tip attachment since M13 adsorption to F, R1-19 and R100-1 pili blocks transmission of these

plasmids equally well (Meynell, 1978). In addition, variation in pilin sequence does not seem to dictate f1 phage attachment or infection because the N-terminus sequences of pilin are not exposed at the tip; as well, the pED208 plasmid which synthesizes the most variant pilin, and the distantly related IncD plasmid, R711b, both plate f1 very efficiently (Frost *et al.*, 1986; Worobec *et al.*, 1986; Lawn *et al.*, 1967; Bradley, 1977). Instead, the differences in pilus number or assembly/retraction kinetics were thought to affect f1 sensitivity (Ippen-Ihler and Minkley, 1986). A highly conserved hexapeptide, $V_{15}KATFG_{21}$ in F-like pilin (Fig. 6), has been implicated as a possible receptor site for f1 (Ippen-Ihler and Minkley, 1989; Finlay *et al.*, 1984). The possibility that the configuration of pilin subunits at the tip might be different than that in the shaft such that new sites on the pilin subunit are exposed at the tip has also been suggested (Frost, 1993). However, the absence of mutations in pilin that specifically alter f1 attachment has complicated the identification of pilin residues that are important for this function (Burke *et al.*, 1979). The presence of a minor pilus protein at the tip which might be the phage receptor has also been suggested to explain the wide host range of these phages (Frost, 1993).

Additional differences between ColB2 and F pili exist. Unlike F, which produces 1-2 pili per cell, cells carrying ColB2 on average produce 5-10 pili per cell (Finlay *et al.*, 1984). In addition, ColB2 pili do not retract in the presence of cyanide (Finlay *et al.*, 1984; Frost *et al.*, 1985). It is unlikely that these differences can be accounted for by the changes in the pilin sequence, rather an alteration in the underlying mechanism of assembly-disassembly of ColB2 pili might be responsible (Finlay *et al.*, 1984).

1.5 Role of the Pilus in Conjugation

The essential nature of F pili in conjugation has been well documented since mutations preventing the synthesis of pili also inhibit DNA transfer. In addition, binding of pilus-specific antisera and phages, and treatments which remove F pili from the cell surface result in the loss of donor activity (Harden and Meynell, 1972; Novotny *et al.*, 1968, 1969; Tomoeda *et al.*, 1972). Two distinct roles for the pilus in conjugation have been proposed; the foremost being its role in the identification of a suitable recipient cell to establish a mating

contact while a secondary role is in the transport of DNA itself (Ippen-Ihler and Maneewannakul, 1991). In addition, the pilus may also interact with the surface exclusion protein, TraT, on the outer membrane of other F' donor cells in order to reduce redundant DNA transfer (Minkley and Willetts, 1984).

1.5.1 Role of the pilus in conjugation: conduction vs retraction

The concept of the F pilus as a conduit for the unidirectional transfer of DNA was first put forth by Brinton (1971) who also coined the term 'epiviruses' to describe sex pili as filamentous infectious particles containing ssDNA that are obligatorily associated with their host cells. Electron microscopic observation of a donor-recipient pair connected by an extended pilus led Brinton to propose the pilus conduction model in which a ssDNA is transferred along the length of the pilus to the recipient (Brinton, 1971). In support of this model Ou and Anderson (1970) established that DNA transfer occurred between donor and recipient cells held apart by a micromanipulator and most recently, Harrington and Rogerson (1990) demonstrated that low levels of DNA transfer appeared to occur through extended pili in the absence of wall-to-wall contact between donor and recipient cells separated by Nuclepore filters.

Although the lumen of the pilus is large enough to accommodate a ssDNA molecule, nucleic acid was never found to be associated with the pilus (Jacobson 1972). Thus, even though transport of DNA through the pilus lumen might be possible, the crucial role of the pilus in conjugation may be to establish an union with the recipient by acting as a 'grappling hook', then retracting to bring the mating cells into close physical contact, thereby enabling the formation of mating aggregates. This concept of pilus retraction, proposed by Curtiss (1969) and Marvin and Hohn (1969), has gradually replaced the pilus conduction model since retraction best explains the dual role of the pilus in conjugation and phage infection. In support of the pilus retraction model, Jacobson (1972) observed a direct correlation between the decrease in the median length of F pili on cells infected with f1 phage with the concomitant accumulation of phage particles on the cell surface. Achtman's findings that mating mixtures contained more aggregated cells in wall-to-wall contact than mating pairs

connected by an extended pilus, strengthened the notion that the pilus is required only at the initial stage of conjugation (Achtman, 1975; Achtman *et al.*, 1978a). Consistent with this finding, Panicker and Minkley (1985) and Frost and Simon (1993) demonstrated that the addition of SDS which depolymerizes pili, did not prevent DNA transfer when added to mating mixtures after the aggregates were formed. However, pre-treatment of donor cells with the detergent or its addition at the beginning of the mating process initially abolished donor ability indicating that extracellular pili are required only at the initial stages of aggregate formation and that DNA transfer occurs during close cell-to-cell contact. Furthermore, single mating events observed in real time by video-enhanced light microscopy revealed that a donor and a recipient initially separated by a distance were ultimately in direct physical contact. The points of contact were identified as conjugational junctions, the sites at which DNA transfer is presumed to occur (Durrenberger *et al.*, 1991). Thin section analysis of conjugational junctions revealed the presence of an electron-dense material sandwiched between the outer membranes of the donor and recipient cells. Presumably, the electron-dense region is made up of *tra* proteins such as TraN and pilin, and outer membrane components of the recipient such as OmpA and LPS, acting in concert to stabilize the union (Durrenberger *et al.*, 1991; Silverman, 1997).

Although the retraction process itself has never been visualized for conjugative pili, there is evidence indicating that this might be a natural biological phenomenon. Burke *et al.* (1979) isolated uncharacterized piliated mutants of F*lac* that were defective in DNA transfer due to the inability of their pili to retract indicating that retraction is necessary for conjugation. Bradley (1972) demonstrated that the polar pili of *Pseudomonas aeruginosa* involved in twitching motility, retract upon adsorption of RNA phages. Little is known about the mechanics of the retraction process itself although very rapid kinetics are expected since the rate of shortening of *P. aeruginosa* pili was approximately 100 nm/s (Bradley, 1972). Retraction of the F pilus is thought to be initiated by a local conformational change in pilin subunits at the tip upon contact with an F cell or f1 phage (Folkhard *et al.*, 1979). This change, believed to involve a conformational switch in the assembled pilin from an α-helical form to a predominantly β-sheet form, is rapidly transmitted downwards along the pilus

resulting in the net shortening of the length (Folkhard *et al.*, 1979). Consistent with this notion, Durrenberger (1991) observed a rotational movement of recipient cells upon contact with the F pilus believed to be brought about by pilus retraction. The most compelling evidence for the conformational change is the finding that pilin subunits in the terminal knobs are in a planar array as opposed to a helical array in the filament (Frost and Bazett-Jones, 1991).

1.5.2 Role of the F pilus in recipient cell recognition

The tip of the pilus is implicated in recognition of the recipient cell surface since mating pair formation is inhibited by f1 phage attachment (Ippen and Valentine, 1967; Novotny *et al.*, 1968). The nature of recipient cell envelope components has been extensively investigated and a variety of ConF$^-$ mutants which are recipient cells defective in conjugation with F$^+$ donors, have been isolated (Skurray *et al.*, 1974). These mutants were defective as recipients only when matings occurred in liquid cultures; on solid surfaces, their recipient ability was not altered suggesting that surface mating can surmount the defects of ConF$^-$ recipients (Skurray *et al.*, 1974). ConF$^-$ mutants largely fall into two groups, those with lesions affecting the *ompA* gene and those with heptose-deficient lipopolysaccharide (LPS) implicating these two structures as important components of the recipient cell for conjugation in liquid medium (Monner *et al.*, 1971; Skurray *et al.*, 1974). Whether LPS or OmpA is the true receptor for the F pilus has been the subject of much debate due to the inconsistencies in the findings that purified LPS alone or LPS in combination with OmpA had inhibitory effects on conjugation (Lancaster and Chaudhuri, 1978; Schweizer and Henning; 1977; Van Alphen *et al.*, 1977; Havekes *et al.*, 1976; Achtman *et al.*, 1978b). However, since mutants defective in LPS were also abnormal in the level and function of OmpA, conjugation defects in LPS mutants were presumed to be due to OmpA defects. Hence, LPS was thought to be required for the correct insertion of OmpA in the outer membrane which was believed to be the primary receptor for the F pilus (Schweizer *et al.*, 1978, Achtman, 1978b). Consistent with this notion, Manoil and Rosenbusch (1982) demonstrated that direct interaction between OmpA and LPS is needed

for recipient function and that mutations altering a region of OmpA exposed on the cell surface reduce recipient ability (Manoil, 1983).

In contradiction to earlier suggestions that LPS and OmpA are the receptors for the F pilus, Achtman (1978b) observed that purified F pili were able to bind to the surface of ConF recipients defective in either OmpA or LPS. Using Coulter Counter analysis, he further showed that ConF mutants were severely impaired in mating aggregate formation. This led him to propose that LPS and OmpA might not be the receptors recognized by the F pilus, rather they might be components of the recipient cell required for stabilization of mating aggregates after the formation of wall-to-wall contacts (Achtman, 1978b). This suggestion is consistent with the findings that both LPS and OmpA are dispensable during surface matings where the cells are already in close contact.

Unlike F, the R100-1 plasmid transfers at normal levels to recipients defective in OmpA indicating that its transfer system may use a different cellular protein for mating aggregate stabilization (Skurray *et al.*, 1974; Havekes *et al.*, 1977; Achtman, 1977). ConR100-1$^-$ mutants which are recipient cells defective in conjugation with R100-1$^+$ donors, were found to have no detectable perturbation in their cell envelope (Havekes *et al.*, 1977), suggesting that specific but subtle alterations, perhaps in the LPS may affect the transfer of R100-1.

1.5.3 Role of the pilus in surface exclusion

Almost all conjugative plasmids have evolved a surface exclusion mechanism which inhibits the transfer of DNA to another donor cell harbouring an identical plasmid in order to prevent the metabolic cost of continuous and deleterious donor recipient activity (Firth *et al.*, 1996). In the F-like plasmids, surface exclusion is brought about by the concerted actions of TraS and TraT which are responsible for independent aspects of the phenomenon; together, they cause several thousand-fold reduction in DNA transfer (Achtman *et al.*, 1977; 1980). TraS is a 16.9 kDa inner membrane protein which when present in the recipient, reduces DNA transfer by several hundreds-fold. Its action appears to inhibit triggering of donor conjugal DNA metabolism presumably by blocking the transmission of the mating signal (Frost *et al.*,

1994). The presence of TraT in the outer membrane of recipient cells seems to interfere with an extracellular step in the formation of mating aggregates (Achtman *et al.*, 1980). This 26 kDa lipoprotein, present at an estimated 20 000-30 000 copies per cell, markedly reduces the formation of stable mating aggregates but diminishes transfer levels by only 10-20 fold (Achtman *et al.*, 1980). Based on the specificity of the surface exclusion system, Willetts and Maule (1986) have categorized the F-like plasmids into four surface exclusion groups (SfxI-IV). Accordingly, inhibition of plasmid transfer only occurs when the surface exclusion system in the recipient has the same specificity as the incoming plasmid. It is believed that both TraS and TraT impart specificity in distinguishing between cells carrying plasmids of different surface exclusion groups (Harrison *et al.*, 1992), but the precise nature of this process is unclear. Meynell and Ewins (1973) however, suggested that the specificity comes about by matching the pilus with the homologous surface exclusion proteins. Minkley and Willetts (1984) observed that the transfer efficiency of the F plasmid was reduced when purified F TraT was added to F*lac* donor cells *in vitro*. They subsequently proposed that TraT interacts with the pilus tip and competitively inhibits the binding of the tip to its normal receptor on recipient cell surface, thereby reducing the frequency of functional pairing. Thus, the specificity in TraT-pilus tip interaction is believed to lie in the recognition of the homologous pilin subunit by TraT in a sequence-specific manner (Harrison *et al.*, 1992). Consistent with this notion, members of each surface exclusion group almost always share the same conjugative pilus types as determined by the sequence at the N-terminus of pilin (Willetts and Maule, 1986). For instance, the F plasmid which specifies pilus type I, belongs to SfxI, while ColB2 specifies pilus type II and belongs to SfxII, and R100-1 which expresses a type IV pilus belongs to SfxIV (Willetts and Maule, 1986). Sequence comparisons of TraT proteins from F, ColB2 and R100-1 revealed single amino acid alterations in a five-amino acid region (residues 116-120) important for specificity determination (Harrison *et al.*, 1992). A single Gly_{120}→Ala transition in R100-1 and Asn_{116}→Ser and Gly_{120}→Ala changes in ColB2 seem to be responsible for their TraT specificities (Harrison *et al.*, 1992). Although the differences are quite subtle, Harrison *et al.* (1992) suggested that their effects may be amplified by the oligomeric nature of TraT. They also postulated that since pilin subunits are

related by a five-fold rotational axis, TraT in its pentameric form (Minkley, unpublished results) may interact in a symmetry match with the pilus tip. Accordingly, the central region of the pentamer exposed on the cell surface may constitute the binding site for the pilus tip and would contain the specificity-defining side-chains of critical residues. However, since the direct binding of TraT to the pilus tip was never demonstrated *in vitro*, a different pathway in which TraT acts by masking a region of OmpA that would normally be exposed on the cell surface has also been proposed (Riede and Eschbach, 1986).

1.6 Role of the pilus in bacteriophage infection

As mentioned in previous sections two morphologically distinct groups of bacteriophages have been instrumental in the discovery and characterization of F-like conjugative pili. The spherical RNA and the filamentous DNA phages still define the majority of bacteriophages that use conjugative pili as receptors (Frost, 1993). Although much information on the biology of these phages has been acquired, their infection process still remains a mystery partly due to the lack of knowledge on the precise physiological role of conjugative pili. In what follows, the adsorption and penetration processes of representative phages from each class are discussed.

1.6.1 Spherical RNA phages

The RNA phages which infect an F-piliated *E. coli* cell include R17, MS2, f2 and Qβ (Frost, 1993). The R17 phage which has been studied in detail (Paranchych, 1975) contains a single-stranded RNA molecule of 3 500-4 700 bases enclosed in an icosahedral shell of 180 copies of a coat protein with a single copy of an attachment protein (A protein) exposed on the surface of the virion (Frost, 1993). The R17 phage binds to the lateral surface of F pili and the specific pilin residues important for its attachment have been previously discussed (Section 1.3.8). Reversible attachment of phage particles to both free and cell-associated pili mediated by the A protein has been observed to occur between 4°C-37°C and at an ionic strength of at least 0.1 M. Subsequent release of the A protein-RNA complex is closely coupled to the energetics of the cell, hence occurs only on a cell-attached pilus at 37°C

(Paranchych, 1975). It is believed that upon phage adsorption, a signal transmitted by the pilus somehow catalyzes the cleavage of the A protein and this is followed by active extrusion of the A protein-RNA complex from the phage particle onto the pilus surface. In the presence of divalent cations, this complex is transported along the F pilus and through the cell membrane via pilus retraction. Subsequent internalization of the RNA requires a functional *traD* product which is thought to act as a nucleic acid pump to actively transport ssDNA across the cell envelope (Paranchych, 1975). Thus, the transport of phage RNA may occur in an analogous manner to conjugative DNA transfer except in the reverse direction.

1.6.2 Ff filamentous DNA phages

The filamentous bacteriophages, f1, fd and M13, collectively known as the Ff phages, are among the best characterized phages that infect *E. coli* cells expressing F pili (Rasched and Oberer, 1986). The virion contains a 6 407-nucleotide single-stranded, covalently closed genome encased in a long cylinder composed of one major and four minor coat proteins (Russel, 1991). The virion contains approximately 2 700 monomers of the major coat protein, pVIII, arranged in a pattern of helical 'fish scales' along the length of the filament; five copies each of the minor coat proteins, pVII and pIX are located at one end of the phage particle, while approximately the same number of pIII and pVI minor coat proteins occupy the opposite end of the filament (Webster, 1996; Russel, 1991; Model and Russel, 1988). The sequential two-step infection process is initiated by the specific binding of pIII protein to the tip of the F pilus (Webster, 1996). A maximum of three phage particles can attach simultaneously to the pilus tip indicating the presence of at least three receptor sites (Marvin and Hohn, 1969). Retraction of the pilus immediately following attachment is believed to transport the phage particles to the cell surface (Webster, 1996). The role of the pilus as merely a vehicle by which the phages access a second receptor on the cell surface is evident by their ability to infect F cells at a low frequency (Russel *et al.*, 1988). The second stage of the infection process involves the entry of the entire virion into the host cell (Russel, 1991). This requires the products of the bacterial *tolQ*, *tolR* and *tolA* genes which are found at the 'contact sites' between the inner and outer membranes and are believed to form a complex

linking the two membranes (Webster, 1991). Translocation of the virion requires direct interaction in the periplasm between pIII and the C-terminus of TolA (Riechmann and Holliger, 1997; Click and Webster, 1997).

The pIII protein is by far the best characterized moiety that is known to interact with the F pilus tip. The 406-residue mature pIII arising after the removal of an 18-aa signal sequence, is anchored in the inner membrane via a C-terminal tail with the remaining 379 residues comprising the N-terminus, in the periplasm (Webster, 1996). Three functional domains each separated by glycine-rich repeats, have been identified by deletion analysis (Stengele *et al.*, 1990). The C-terminal membrane anchor presumably anchors pIII to the phage capsid via its interaction with pVI while the two N-terminal domains appear to be directly involved in the infection process since mutations affecting the latter two domains render the phage particle non-infectious (Stengele *et al.*, 1990). In the electron microscope, the N-terminal part of pIII can be seen protruding as a knob-like structure at one end of the virion (Gray *et al.*, 1981). This region can be proteolytically removed by subtilisin digestion as a highly soluble 36 kDa form capable of F pilus binding (Webster, 1996). Further analysis of this fragment identified residues 99-196 (D2) as essential for pilus binding and residues 53-107 (D1) as important for penetration of the host, presumably by specific interactions with the TolQRA complex (Webster, 1996; Stengele *et al.*, 1990). Recently, Reichmann and Holliger (1997) revealed that the D1 and D2 domains are normally in close association and that this association is somewhat relieved by the binding of Domain II to the pilus tip which then unveils a region in Domain I that binds TolA.

1.7 F pilus assembly

Despite the rather simple nature of the F pilus, its assembly requires the co-ordinate expression of at least fifteen *tra* gene products. As discussed in Section 1.4, the propilin structural gene, *traA*, and the products of *traQ* and *traX* involved in its maturation, accomplish the synthesis of membrane F pilin. Mutations which affect the synthesis of *traL, traE, traK, traB, traV, traC, traW, traU, traF, traH, trbC* or the N-terminal region of *traG* affect piliation and result in the accumulation of membrane F pilin. As such, the products of these genes are

presumed to be involved in the polymerization of pilin into the pilus filament (reviewed in Frost *et al.*, 1994).

A summary of the known or predicted characteristics of these proteins is included in Table 1.1. Although little is known about the precise functions of each of these proteins, subcellular localization studies of some of these gene products suggest that they might be associated with the cell envelope (Frost *et al.*, 1994). In addition, cell fractionation studies to determine the location of specific individual *tra* proteins gave inconsistent results when these proteins were synthesized in isolation as compared to when synthesized in the presence of other *tra* products, suggesting protein-protein interactions among the gene products involved in pilus assembly (Firth *et al.*, 1996). Based on this premise, pilus assembly proteins might form a multiprotein complex connecting the inner and outer membranes and facilitate the polymerization and elaboration of the pilus through the cell envelope (Firth *et al.*, 1996).

Analysis of the gene products in Table 1.1 identify TraF, TraU, TraW and TrbC to be periplasmically located; their precursor forms contain typical N-terminal peptidase I signal sequences and their cleavage and translocation to the periplasm have been experimentally demonstrated (Moore *et al.*, 1990; Maneewannakul *et al.*, 1991;1992; Wu *et al.*, 1988). Likewise, the gene products of *traK* and *traH* also contain similar N-terminal peptidase I signal sequences and hence they are also predicted to be located in the periplasm (Penfold *et al.*, 1994; Ham *et al.*, 1989). An ATP/GTP binding motif is present in TraH but its relevance to pilus assembly has not been determined (Frost *et al.*, 1994). Insertion mutations which eliminate *trbC* expression give rise to an apiliate phenotype that still retains slight sensitivity to f1 phage suggesting the availability of an unextended pilus tip structure on the cell surface (Maneewannakul *et al.*, 1991). Donor cells carrying insertion mutations in *traU* are extremely transfer-deficient but express reduced numbers of pili that seem to be defective in pilus-specific phage infection (Moore *et al.*, 1990). Characteristic of bacterial outer membrane lipoproteins, the product of *traV* undergoes signal peptidase II processing. Presumably a covalent lipid modification results in the apparent increase in the molecular weight of the mature form of TraV (Doran *et al.*, 1994). TraV is the only lipoprotein required for pilus assembly and as such, is likely to be tethered to the inner leaflet of the

Table 1.1 Characteristics of *tra* and *trb* gene products required for pilus assembly[a]

Gene	Product length (aa)[b]	Product size (kDa)[b]	Product location[c]
traB	475	50.460	IM (pred)
traC	875	99.191	C (M) (pred); IM (exp)
traE	188	21.161	IM (pred); IM (exp)
traF	247 [228]	27.973 [25.939]	P/OM (pred); P (exp)
traG	938 [487]	102.471[52.719]	IM/P(pred); IM/P (exp)
traH	458 [434-435]	50.244 [47.781-47.851]	P/OM (pred)
traK	242 [221]	25.621 [23.307]	P/OM (pred)
traL	91	10.379	C (M) (pred)
traU	330 [308]	36.786 [34.275]	P/OM (pred); P (exp)
traV	171[153]	18.588 [16.564]	OM (pred)
traW	210 [192-193]	23.630 [21.690-21.761]	P/OM (pred); P (exp)
trbC	212 [191]	23.433 [21.226]	P/OM (pred); P (exp)

[a] Data obtained from Frost *et al.*, 1994.

[b] Sizes and lengths are calculated from the deduced amino acid of each product. Sizes and lengths of processed products are shown in brackets.

[c] Location of products as determined experimentally (exp) or as predicted (pred) by hydropathy analysis. C, cytoplasm; IM, inner membrane; OM, outer membrane; P, periplasm; (M), peripheral membrane association.

outer membrane via its lipid moiety to mediate interactions with cell wall peptidoglycan (Doran *et al*, 1994).

Hydropathy analyses in combination with cellular fractionation studies identified the products of *traB* and *traG* as integral inner membrane proteins (Moore *et al.*, 1987; Manning *et al.*, 1981; Frost *et al.*, 1994). TraB is presumably anchored to the inner membrane at its N-terminus with the rest of the protein in the periplasm. Its proline and glycine rich nature is characteristic of proteins with extended conformation such as TonB and TolA (Frost *et al.*, 1994). The gene product of *traG* is a unique bifunctional protein that couples pilus synthesis with mating aggregate stabilization (Achtman *et al.*, 1971). Although all characterized mutations in *traG* result in transfer deficiency, only the first 534 of the 938 residues of TraG are important for pilus synthesis (Firth and Skurray, 1992). Mutations or deletion of the C-terminal region do not affect piliation, however, its importance in mating aggregate stabilization is reflected by severe transfer deficiency and failure of the cells to exist in stable aggregates with F cells (Manning *et al.*, 1981; Firth and Skurray, 1992). Analysis of TraC revealed a cytoplasmic location when synthesized in isolation but appeared to fractionate with the inner membrane when other *tra* products are present suggesting that TraC normally associates with one or more inner membrane proteins (Schandel *et al.*, 1992). It contains a signature sequence for a Type A ATP/GTP binding site, however, its significance to the energetics of pilus assembly if any, is yet to be experimentally proven (Frost *et al.*, 1994). Although all *traC* mutations prevent pilus outgrowth, the *traC1044* missense mutation which contains a $Arg_{111} \rightarrow$ Cys substitution, appears to cause only partial defects in mating aggregate formation and f1 infection despite the obvious absence of pili on the cell surface (Schandel *et al.*, 1987; 1990). Thus, it seems that although the product of *traC1044* is able to participate in the formation of a transmembrane complex with an F pilus tip exposed on the cell surface, it is unable to polymerize pilin subunits into an extended filament (Schandel *et al.*, 1987).

Attempts to visualize the product of *traL* using an overexpression system were not successful but based on hydropathy analysis TraL seems to be peripherally associated with the inner membrane (Laine *et al.*, 1985; Frost *et al.*, 1994). The product of *traE* fractionates

with the inner membrane and is predicted to be anchored in the membrane via its N-terminus (Laine *et al.*, 1985; Frost *et al.*, 1994).

Apart from the twelve proteins essential for pilus function and assembly, a few other *tra* gene products have been identified which seem to act as accessory elements in the assembly process since mutations in these genes normally do not cause a discernible effect in the conjugative function of the pilus. For instance, both mutations preventing the synthesis of TrbI or the overproduction of the polypeptide lower the plating efficiency of pilus-specific phages (Maneewannakul *et al.*, 1992). In the absence of TraP, mating efficiency is reduced by 100-fold; in addition, these cells synthesize unusually long pili which appear to be weakly attached to the cell (Anthony *et al.*, 1996).

In the presence of a full complement of functional pilus assembly proteins, approximately 15% of pilin from the inner membrane pool is polymerized into the pilus filament (Silverman, 1987). Electron microscopy of thin sections revealed that pili emerge from adhesion zones or Bayer junctions, where the inner and outer membranes are contiguous (Bayer, 1991). Although the existence and nature of Bayer junctions still remain controversial, the distribution of pilus assembly proteins in the cell envelope strongly supports the contention that these proteins may interact with each other and pilin to form a transenvelope connection and provide a site for pilus extension and perhaps a route for DNA transfer (Firth *et al.*, 1996). Pilus assembly is halted in the presence of respiratory poisons indicating an absolute requirement for energy for this process (Section 1.3.4). TraC and TraH which contain nucleotide binding motifs, could drive the assembly process by providing the needed energy in the form of high energy phosphates via hydrolysis of ATP/GTP. The mechanism of F pilus outgrowth has not been studied and whether pilin subunits are added at the tip or base during polymerization is unknown. Although the possibility that pilus extension, analogous to flagella outgrowth, may occur by addition of new subunits at the tip has been proposed, recent evidence indicates that the thick, flexible IncHI1 conjugative pili lengthen by subunit addition at the base suggesting a similar mechanism for F pilus outgrowth (Silverman, 1987; Maher *et al.*, 1993).

1.7.1 A transporter for T-DNA transfer in *Agrobacterium tumefaciens* and a model for F pilus assembly

Over the past few years a vast body of evidence has been gathered on the nature of the T-DNA transfer mediated by the Ti plasmid of *Agrobacterium tumefaciens*. T-DNA transfer shares remarkable similarities with the F plasmid conjugation system both in the processing and transmission of a ssDNA molecule, as well as in the absolute requirement for an extracellular pilus (Lessl and Lanka, 1994; Fullner *et al.*, 1996). Both short, rigid and long, flexible pili have been detected on the surface of *Agrobacterium tumefaciens* cells harbouring the Ti plasmid, however, only the long flexible pili have been shown to be essential for mobilization of DNA-protein complexes (Kado, 1994; Fullner *et al.*, 1996). The products of the *virB* operon required for pilus synthesis have been extensively studied and a model for the formation of a transport complex involved in the polymerization of pilin and DNA transfer has been postulated (Shirasu *et al.*, 1990; Christie, 1997; Baron and Zambryski, 1996; Baker *et al.*, 1997). Since homologues of *virB* gene products have been identified in the F system and the characteristics of their proteins are remarkably similar, the T-DNA transport complex may be envisaged as a model system for the assembly of the F pilus (Firth *et al.*, 1996).

Twelve genes have been identified which specify protein products that are involved in pilus formation; eleven of these, VirB2-VirB11 and VirD4 are essential core components of either the pilus or the assembly machinery while VirB1 seems to be auxiliary for T-DNA transport (Shirasu *et al.*, 1990; Fullner *et al.*, 1996; Christie, 1997; Berger and Christie, 1994). The structural protein of the pilus is yet to be determined but the gene product of *virB2* has been implicated as the pilin subunit based on its sequence similarity with F *traA* (Kado, 1994). Like propilin, VirB2 is proteolytically processed from a 12.3 kDa precursor to a 7.2 kDa mature inner membrane protein by removal of a 48 aa signal sequence (Jones *et al.*, 1996). Circumstantial evidence also suggests that VirB1*, the C-terminal product of VirB1, and VirB^5 may also form structural components of the pilus (Baron and Zambryski, 1996). Analogous to the F pilus assembly system, most of the *virB* gene products also fractionate with both membranes congruent with the view that these proteins may assemble to form a

membrane spanning channel for the translocation of DNA as well as proteins (Shirasu *et al*, 1990; Christie, 1997). Detailed biochemical and genetic analyses of *virB* proteins revealed intimate protein-protein interactions among several of the proteins and helped define the early stages in the assembly of a conjugation apparatus (Christie, 1997). The mechanism of assembly seems to be initiated by the association of three proteins, VirD4, VirB4 and VirB11, with the inner membrane (Christie, 1997). All three proteins contain ATP/GTP binding motifs with the ATPase activity of VirB4 and VirB11 being essential for DNA transfer and, as such, may energize the assembly process (Christie, 1997). Interestingly, the F plasmid TraC and TraD proteins which are homologues of VirB4 and VirD4 respectively, also contain signature sequences for nucleotide binding (Firth *et al.*, 1996; Frost *et al.*, 1994). Similar to TraV, VirB7 is the only lipoprotein in the *virB* operon and it has been shown to form covalently linked heterodimers with the periplasmically located VirB9 (Baron *et al.*, 1997a). This interaction is believed to anchor the transport complex to the outer membrane (Christie, 1997). Formation of VirB7-VirB9 heterodimers is critical for the stabilization of VirB9 as steady state levels of VirB9 are dramatically reduced in a *virB7* mutant (Baron *et al.*, 1997a). Further, the heterodimer is believed to interact with the inner membrane-associated VirB10, a TraB homologue, and allow its multimerization (Baron *et al.*, 1997a; Firth *et al.*, 1996). In addition, the absence of VirB7 and VirB9 synthesis correlates with a reduction in the stable accumulation of several other *virB* proteins including VirB3 and VirB5 which are the homologues of TraL and TraE, respectively (Baron *et al.*, 1997a; Firth *et al.*, 1996). These findings suggest that the VirB7-VirB9 heterodimer may play a critical role as a nucleation centre for the assembly of the transport complex as well as stabilizing other *virB* proteins during biogenesis of the transporter complex (Christie, 1997). In contrast to other *virB* proteins, mutations affecting the synthesis of VirB1 result in a decrease in DNA transfer (Berger and Christie, 1994). VirB1 undergoes proteolytic processing to give rise to a C-terminal-secreted product, VirB1* which is found in loose association with the cell surface (Baron *et al.*, 1997b). Further, the recent finding that the N-terminal region of VirB1 might be a lytic transglycosylase suggests that VirB1 might facilitate the assembly of the T-complex transport apparatus by causing localized lysis of the peptidoglycan (Baron *et al.*, 1997b).

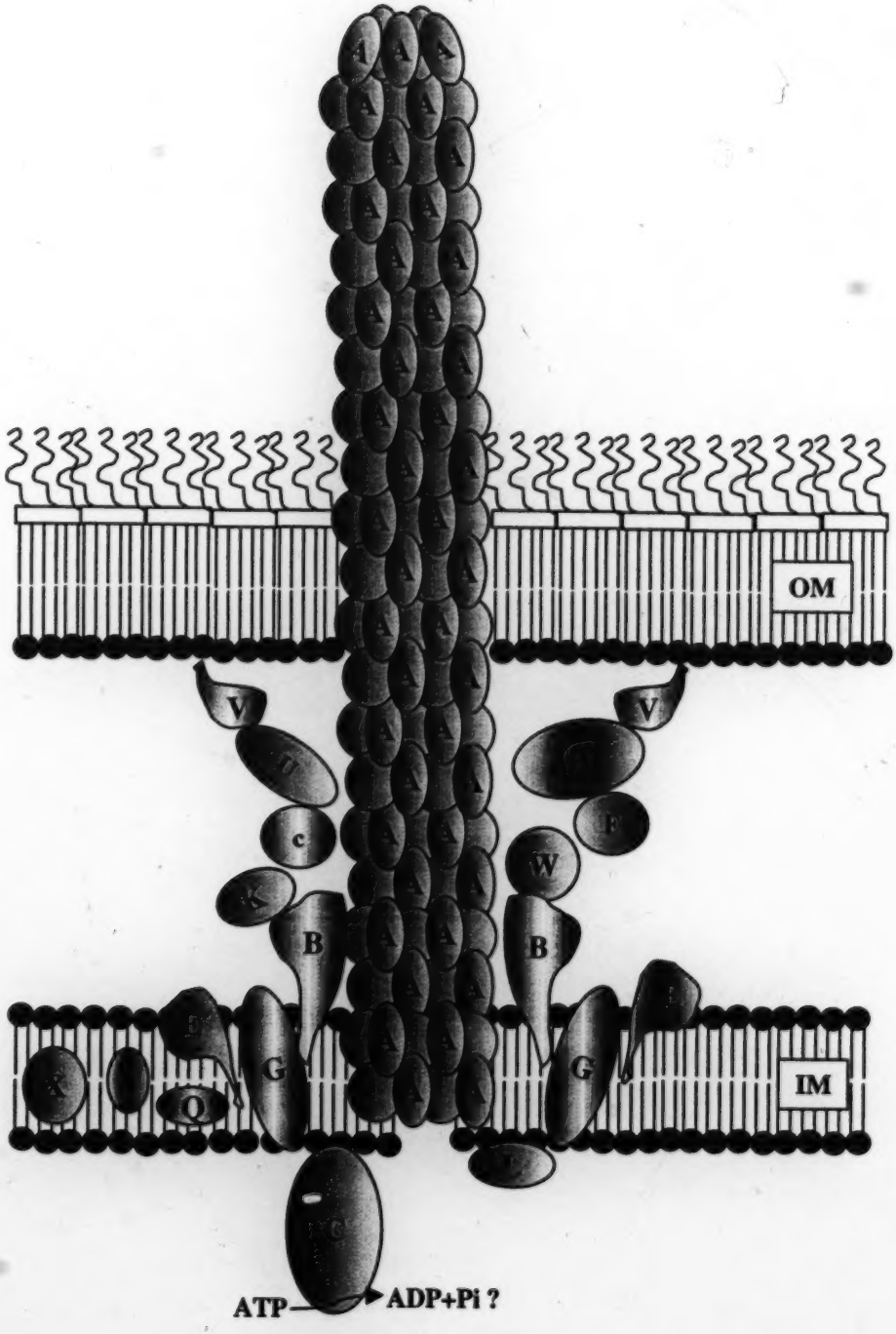
Interestingly, the products of *orf169* in F, *gene 19* of R1 and *trbN* of the broad host range plasmid, RP4, also show homology to proteins of the lytic transglycosylase family (Firth *et al.*, 1996).

Based on the similarities among the pilus assembly proteins, the picture that is emerging for the assembly of the F pilus is one that is reminiscent of the T-complex transport apparatus itself, where all or the majority of the assembly proteins form a multi-protein channel through which pilin is polymerized and DNA is transported (Fig. 1.7). In this respect, F-plasmid-mediated conjugation or Gram-negative conjugation in general, has been viewed as a specialized form of macromolecular transport whereby both protein and DNA are translocated across two bacterial membranes (Firth *et al.*, 1996). Evolutionary relationships among *virB*, *tra* proteins of F and the broad host range plasmids IncN and IncP, with the *ptl* proteins responsible for the export of *Bordetella pertussis* toxin, further lend credence to the above notion (Winans *et al.*, 1996; Firth *et al.*, 1994). In fact, pilus-mediated conjugation has been classified as a type IV secretion system dedicated for the transmission of DNA to recipient cells in a process requiring cellular contact (Christie, 1997). However, the DNA transfer mediated by the F plasmid may be a unique type IV secretion system since the transfer process also requires subunit disassembly via pilus retraction and furthermore the pilus itself, or a vestige thereof remaining after retraction, may form part of the mating channel (Firth *et al.*, 1996).

1.8 Scope of this thesis

The F plasmid-mediated DNA transfer process has been the subject of investigation for several decades. However, many aspects of the conjugation process still remain to be elucidated. In particular, a detailed knowledge of the biogenesis and function of F pili has been elusive despite the fact that these appendages represent the most conspicuous characteristic of cells harbouring this plasmid. The studies described here were performed with the broad aim of gaining further understanding of the nature of this cell-surface structure and hence the conjugative process encoded by F itself.

Fig. 1.7 A speculative model for F pilus assembly. The figure depicts the polymerization of pilin from the inner membrane (IM) through the outer membrane (OM) mediated by an array of pilus assembly proteins. Cellular locations of the *tra* proteins are as described in Frost *et al.*, 1994 and in Table 1.1. TraV lipoprotein which is shown tethered to the inner leaflet of the outer membrane, might anchor the transmembrane complex formed from the association of *tra* proteins located in the periplasm and the inner membrane to the peptidoglycan. Hydrolysis of ATP by TraC may energize the assembly process. Interactions among any of the *tra* products shown here have not been been proven experimentally.


A
OM
V
U
c
K
B
W
F
G
X
Q
IM
C
ATP
ADP+Pi ?

The specific goal of this thesis is to establish a genetic or structural basis for the variation exhibited by F-like pili in recipient cell recognition, f1 phage infection and surface exclusion. The subject was addressed by first determining the contribution of the pilin sequence, specifically the differences at the N-terminus, to specificities in pilus-related functions. Pilin genes from F, ColB2 and R100-1 plasmids were used to complement an F *traA* null mutation and the pilus-related properties were assessed. The results, presented in Chapter 2 reveal the surprising finding that the pilin sequence does not determine specificities noted in pilus function and suggests the possibilities that the pilin subunit of F-like plasmids may be processed or modified differently, or that the presence of one or more minor proteins at the pilus tip may define the specificities. In order to identify candidate *tra* genes involved in these processes, the DNA sequence of the pilus synthesis genes from the R100-1 plasmid was determined in order to establish the extent of sequence identity with the F plasmid. Sequence comparisons of gene products of *traL, E, K, B* and *P* together with an analysis of TraP as the possible specificity determinant are presented in Chapter 3. Chapter 4 describes the studies performed to isolate the putative pilus tip protein by physical means using a fusion protein derived from the pIII protein of M13 phage. The findings from this work question the presence of a tip protein and raise alternative possibilities to explain the nature of the phage receptor at the pilus tip. Chapter 6 describes the results of trans-complementation studies between F and R100-1 pilus synthesis genes and offers some new insights into the nature of these proteins between the two plasmids as well as a possible explanation for the phenotypic differences of their pili.

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CHAPTER 2

Does the pilin sequence determine the specificity of pilus function?*

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2.1 INTRODUCTION

Despite their functional and morphological similarities, the conjugative pili of F and F-like plasmids, ColB2 and R100-1 (Section 1.2.3), exhibit subtle variations in recognition of recipient cells, conferring susceptibility to pilus-specific phage infection and in recognition of TraT during surface exclusion. These specificities have been attributed to the changes in the primary sequence of their pilin proteins, specifically those confined to the N-terminus (Willetts and Maule, 1974, 1986; Frost *et al.*, 1985).

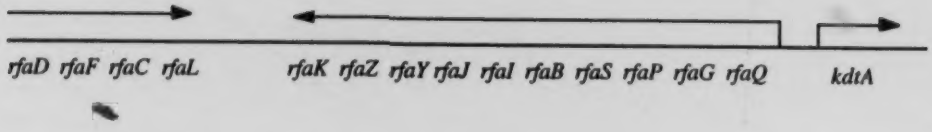
Our recent work with recipient cells carrying lesions in the lipopolysaccharide (LPS) biosynthetic genes (*rfa* genes; Fig. 2.1, Schnaitman and Klena, 1993), has revealed that F, R100-1 and ColB2 plasmids each requires different receptor moieties in the LPS for plasmid transfer (Anthony *et al.*, 1994). The transfer of the F plasmid was greatly reduced by mutations in the *rfaP* locus. Since the gene product of *rfaP* is required for the addition of the pyrophosphorylethanolamine (PPEA) group to Heptose I (Fig. 2.1), these results implicated the PPEA group as the receptor recognized by the F pilus. Although mutations that prevent the extension of LPS beyond the 3-deoxy-D-manno-octulosonic acid (KDO) group (Fig. 2.1) did not prevent R100-1 transfer, the presence of exogenous phosphorylethanolamine (PEA) in the mating mixture inhibited the transfer of this plasmid. This suggested that the R100-1 pilus might recognize a phosphorylated substituent, possibly the PEA group on KDO II (Fig. 2.1). Since mutations that alter the HepII-HepIII (L-glycero-D-mannoheptose) region reduced ColB2 transfer, these two substituents might be the receptor for the ColB2 pilus. Lesions which prevent the synthesis of functional OmpA, reduced the transfer of both F and ColB2 plasmids but not R100-1.

The varying degrees of susceptibility to pilus-specific phage infection conferred by F, ColB2 and R100-1 plasmids have been discussed previously (Sections 1.3.2 and 1.4.5). Also, the basis for the specificity of TraT-mediated surface exclusion has been described in Section 1.5.3.

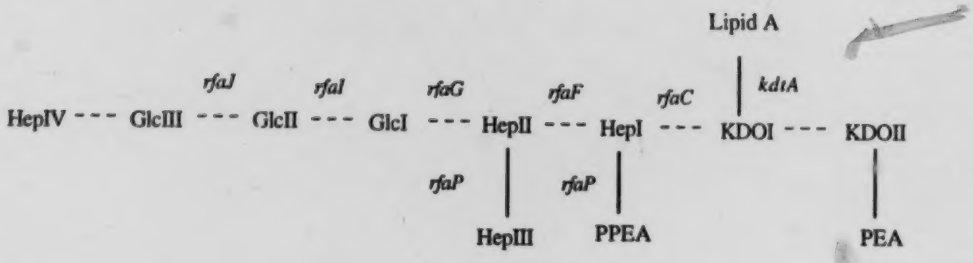
The study described here was undertaken to devise a system which would allow a definitive assessment of the contribution of pilin sequence to specificities in pilus function. For this purpose, a null mutation in *traA* was created by disrupting the gene with a

Fig. 2.1 (A) The order of the genes in the *rfa* region. (B) The structure of the core lipopolysaccharide of *Escherichia coli.* The arrows in (A) indicate the length and direction of mRNA transcripts. Abbreviations: Glc, glucose; Hep, L-glyero-D-mannoheptose; KDO, 3-deoxy-D-manno-octulosonic acid; PPEA, pyrophosphorylethanolamine; PEA, phosphorylethanolamine. These figures are adapted from Klena and Schnaitman (1993).

A


rfaD rfaF rfaC rfaL
rfaK rfaZ rfaY rfaJ rfaI rfaB rfaS rfaP rfaG rfaQ
kdtA

B


Lipid A
rfaJ
rfaI
rfaG
rfaF
rfaC
kdtA
HepIV
GlcIII
GlcII
GlcI
HepII
HepI
KDOI
KDOII
rfaP
rfaP
HepIII
PPEA
PEA

chloramphenicol acetyltransferase (CAT) cassette. Chimeric plasmids expressing either F, ColB2 or R100-1 pilin genes were individually tested for their ability to complement this mutation. Plasmid transfer to recipient cells carrying mutations in various *rfa* and *ompA* loci and to recipients expressing the F TraT protein was measured. In addition, susceptibility to both f1 and R17 phages was determined.

2.2 RESULTS

2.2.1 Construction and phenotypic characterization of pOX38A::CAT

A detailed schematic of the procedure used for constructing an insertion mutation in the pilin gene, *traA*, is presented in Fig. 2.2A and 2.2B. The insertion mutation was first constructed in pLF164 (Amp^R, Table 2.4), a multicopy plasmid which carries a 1.4 kb *Pst*I fragment that extends from the distal end of *traY* to the proximal end of *traK* (F coordinates 2110-3561). A chloramphenicol acetyltransferase (CAT) gene cassette was inserted into the unique *Nae*I site at the junction between the leader sequence and the pilin protein coding sequence (Fig. 2.2A). Recombinant plasmids, pLF164A::CAT, which contained the CAT insertion in the same direction as *traA* transcription were selected by restriction analysis.

This mutation was subsequently crossed onto the self-transmissible F plasmid derivative, pOX38 (Table 2.4), by *in vivo* recombination during a triparental mating (Kathir and Ippen-Ihler, 1991) as shown in Fig. 2.2B. The plasmid pLF164A::CAT was initially transformed into the *rec*$^+$ *E. coli* JC3272 (Table 2.4). The pOX38 plasmid was subsequently mated into this strain to allow *in vivo* recombination between the homologous *tra* regions on the plasmids to occur. A second recipient, *E. coli* XK1200 (Nal^R, Table 2.4), was added to the mating mixture and transconjugants were selected for chloramphenicol and nalidixic acid resistance and ampicillin sensitivity. Restriction analysis of plasmid DNA from these transconjugants confirmed the presence of the CAT insertion in the *traA* gene in pOX38. This construct was named pOX38A::CAT. Western blot analysis of SDS-PAGE (Section 4.4) fractionated proteins of pOX38A::CAT mutant strains with TraA-specific polyclonal

Fig. 2.2A Construction of pLF164A::CAT. The *traA* gene in the plasmid, pLF164, was disrupted with the insertion of a CAT gene cassette at the *Nae*I site as described in Section 2.4. Plasmid DNA isolated from Amp^R and Cm^R transformants was screened for the presence of the insert. Only the chimeric plasmids that contained the CAT cassette inserted in the direction of *traA* transcription was used in the following procedure to generate pOX38A::CAT (Fig. 2B).

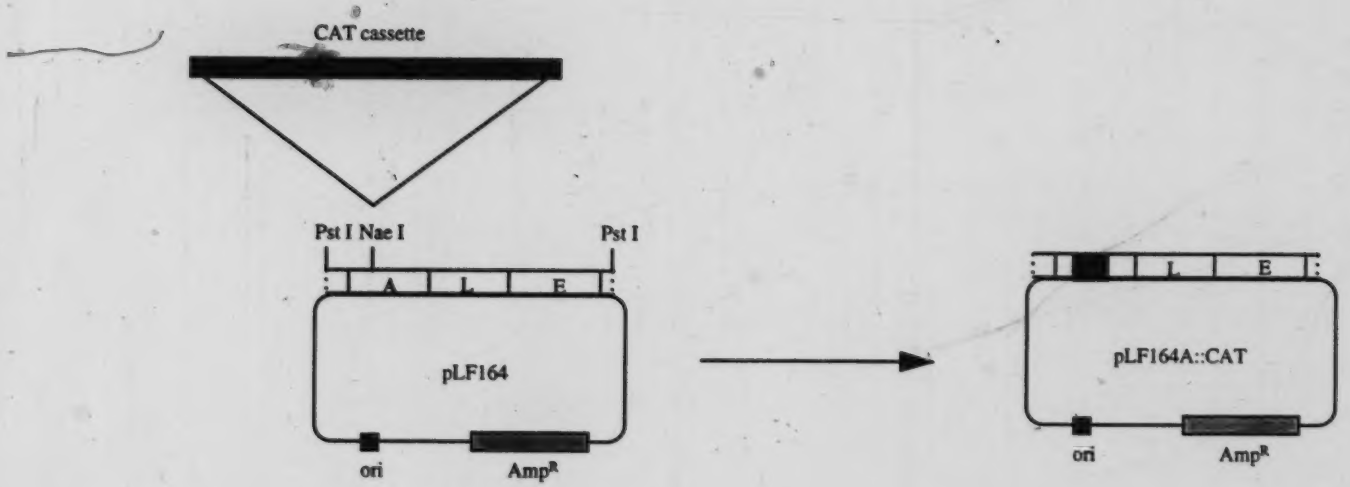
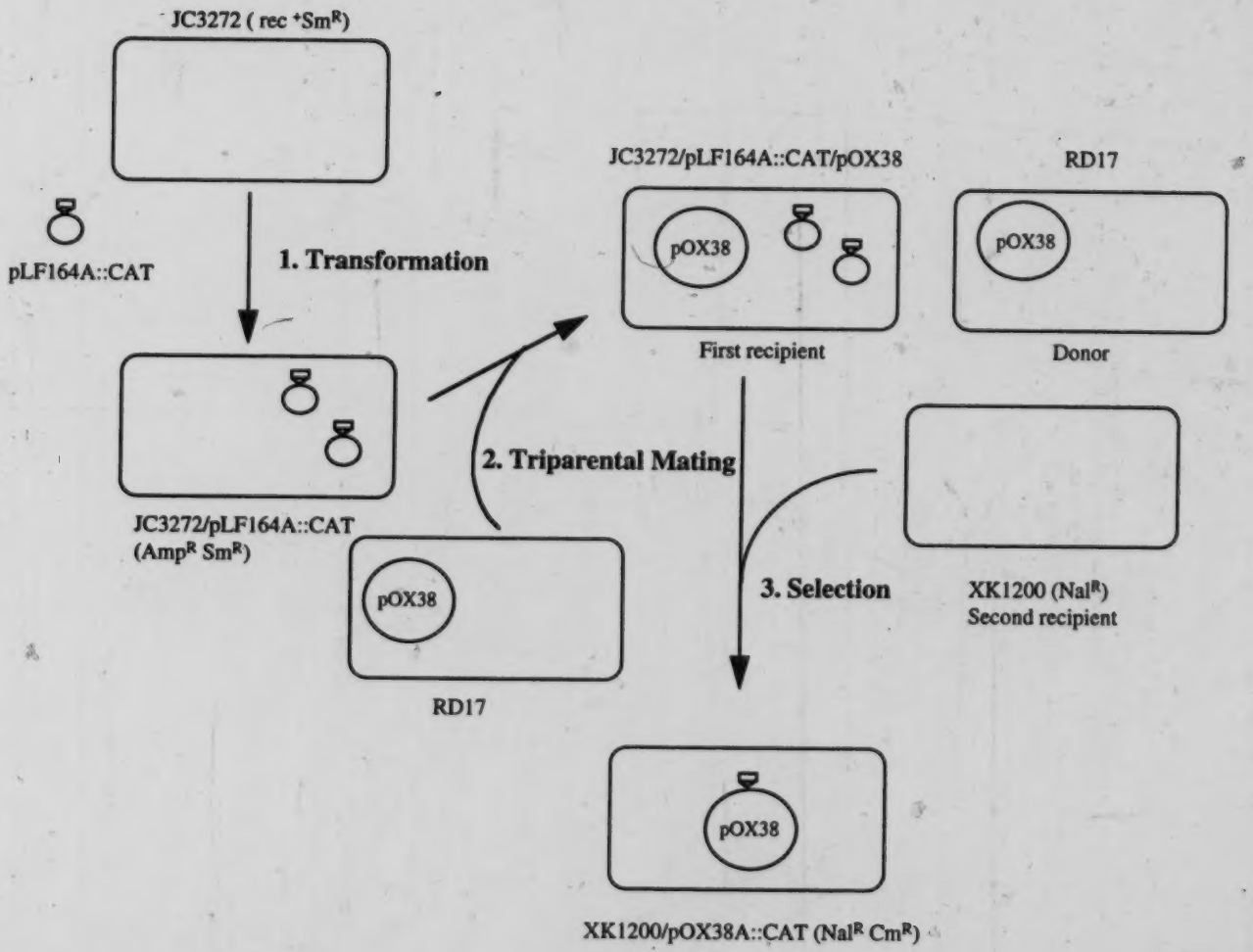

CAT cassette
Pst I Nae I
Pst I
A
L
E
pLF164
ori
Amp^R
L
E
pLF164A::CAT
ori
Amp^R

Fig. 2.2B Construction of pOX38A::CAT. The diagram illustrates the triparental mating procedure used for the generation of pOX38A::CAT. The experimental details are described in Section 2.4 and the genotypes of strains used are listed in Table 2.4.


JC3272 (rec +SmR)
pLF164A::CAT
1. Transformation
JC3272/pLF164A::CAT
(AmpR SmR)
2. Triparental Mating
pOX38
RD17
JC3272/pLF164A::CAT/pOX38
First recipient
RD17
Donor
3. Selection
XK1200 (NalR)
Second recipient
pOX38
XK1200/pOX38A::CAT (NalR CmR)

antiserum confirmed the absence of pilin polypeptides (data not shown). Consistent with this observation the mutant plasmid was deficient in transfer and resistant to f1 and R17 phages. Transmission electron microscopy (TEM, Section 2.4)) revealed that pOX38A::CAT mutant cells did not produce any pili (Table 2.1). However, both pilus production and transfer ability were restored when pLF164 was supplied in trans (Table 2.1) indicating that the CAT insertion is not polar on the expression of downstream genes in the transfer operon.

2.2.2 Complementation analysis of pOX38A::CAT with pilin genes from F, ColB2 or R100-1 plasmids

The ability of chimeric plasmids expressing either F, ColB2 or R100-1 pilin genes to complement the *traA* mutation in pOX38A::CAT was assessed in a mating experiment. Initially, the plasmids pLF164, pBF203 and pLF107 which are derivatives of the pUC8 plasmid carrying the *traALE* region from F, ColB2 and R100-1, respectively (Table 2.4) were transformed into *E. coli* XK1200 cells harbouring pOX38A::CAT (Section 2.4). These transformants were used as donors in mating experiments (Section 2.4) with *E. coli* ED24 (Table 2.4) as the recipient. The mating efficiency for each chimeric plasmid is expressed as the number of transconjugants per 100 donors in Table 2.1. The results indicate that all three plasmids were able to restore transfer ability to pOX38A::CAT; the mating efficiency in the presence of pLF164 was approximately 0.9% while both pBF203 and pLF107 restored the mating efficiency to about 2% of the wild-type plasmid, pOX38-Km. Transconjugants arising from the matings were tested for DNA transfer to confirm that recombination between the pilin genes on the chimeric plasmids and pOX38A::CAT had not occurred.

Next, susceptibility to pilus-specific phage infection was tested in the presence of F, ColB2 and R100-1 pilin genes. XK1200 cells carrying pOX38A::CAT and one of pLF164, pBF203 or pLF107 were infected with either f1 or R17 phages and the extent of cell lysis was qualitatively measured on spot tests (Section 2.4). The results are summarized in Table 2.1. In contrast to the parental plasmids ColB2 and R100-1, cells carrying pBF203 and pOX38A::CAT or pLF107 and pOX38A::CAT were fully susceptible to f1 infection. In

Table 2.1 Characteristics of pOX38A::CAT plasmids expressing either F, ColB2 or R100-1 pilin

Plasmid/s[a]	Transconjugants per 100 donors[b]	Phage sensitivity[c] f1	R17	Phage binding to pili[d] f1	R17
pOX38A::CAT	0	-	-	NA	NA
pOX38A::CAT+					
pLF164 (F)	0.93	+++	+++	yes	yes
pLF107 (R100-1)	2.47	+++	+++	yes	yes
pBF203 (ColB2)	2.10	+++	+++	yes	yes
pOX38-Km	90	+++	+++	yes	yes
R100-1	100	+	-[e]	yes[e]	no
ColB2	100	+	+++[e]	yes[e]	yes[e]

[a] *E. coli* host strain used was XK1200 (Table 2.4).

[b] Recipient for matings was *E. coli* ED24 (Table 2.4).

[c] Phage sensitivity was determined using qualitative spot tests as described in Section 2.4. (+++) fully sensitive; (+) weakly sensitive; (-) resistant

[d] Phage binding to pili was determined by EM as described in Section 2.4; ND refers to not determined.

[e] Information obtained from Meynell (1978), Willetts and Maule (1986) and Finlay *et al.* (1984).

addition, the latter was sensitive to R17 phages even though the R100-1 plasmid is inherently resistant to this phage. Further, analysis by TEM revealed that both f1 and R17 phages attached to the tip and the sides of pili of all three constructs equally well (Table 2.1). These results indicate that pOX38A::CAT expressing either ColB2 or R100-1 pili still retained an F-like pilus-specific phage sensitivity profile.

2.2.3 Effect of switching pilin genes on the requirement for LPS receptor and OmpA in the recipient cell

In order to determine if the requirements for the different LPS moieties and OmpA in the recipient for the transfer of F, ColB2 and R100-1 plasmids, are due to the differences in their pilin protein, the transfer of pOX38A::CAT to recipients defective in *rfaP*, *rfaG* or *ompA* genes was investigated. A complete list of the genotypes of all the strains used in this work is provided in Table 2.4. Mutations in *rfaP* and *rfaG* prevent the addition of PPEA to Heptose I and the addition of Glucose I to Heptose II, respectively (Fig. 2.1). As such, the *rfaP* mutation reduces F plasmid transfer whereas the *rfaG* mutation reduces the transfer of both ColB2 and F due to its polar effect on *rfaP*. Neither mutation has any effect on R100-1 transfer (Anthony *et al.*, 1994). Mutations in *ompA* reduce both F and ColB2 transfer but not R100-1 (Anthony *et al.*, 1994). The results from mating experiments with pOX38A::CAT expressing either F, R100-1 or ColB2 pilin with the above-mentioned recipients are shown in Table 2.2. The CS2198 strain carrying a mutation in *rfaJ* (Table 2.4 and Fig. 2.1) which does not affect recipient ability (Anthony *et al.*, 1994) was used as an isogenic wild type control. In the presence of either pLF164, pLF107 or pBF203, the levels of pOX38A::CAT transfer are reduced to both *rfaP* and *rfaG* recipients similar to the wild-type pOX38-Tc (a derivative of the pOX38 plasmid tagged with a transposon specifying tetracycline resistance; Table 2.4). In addition, the transfer of pOX38A::CAT from donor cells complemented with either F, ColB2 or R100-1 pilin genes is dramatically reduced to recipients carrying the *ompA* mutation (Table 2.4). These results indicate that even though the pilin proteins were different, the transfer of pOX38A::CAT still depended on the presence of the PPEA moiety and OmpA in the recipient.

Table 2.2 The specificity of DNA transfer to *rfa* and *ompA* recipients by pOX38A::CAT complemented with either F, ColB2 or R100-1 pilin gene

Donor *E. coli*	Recipient *E. coli* [a] (relevant phenotype)	Transconjugants per 100 donors	% Control [b, c]
pOX38A:: CAT/ pLF164 (F pilin)	CS2198 (*rfaJ* Km^R)	0.94	100.0
	CS2187 (*rfaG* Km^R)	0.04	4.2
	CS2193 (*rfaP* Km^R)	0.02	2.1
	CS277 (*ompA* Km^R)	0.009	0.95
pOX38A::CAT/ pLF107 (R100-1 pilin)	CS2198 (*rfaJ* Km^R)	1.1	100.0
	CS2187 (*rfaG* Km^R)	0.087	7.8
	CS2193 (*rfaP* Km^R)	0.068	6.1
	CS277 (*ompA* Km^R)	0.003	0.27
pOX38A::CAT/ pBF203 (ColB2 pilin)	CS2198 (*rfaJ* Km^R)	1.07	100.0
	CS2187 (*rfaG* Km^R)	0.06	5.7
	CS2193 (*rfaP* Km^R)	0.04	3.0
	CS277 (*ompA* Km^R)	0.004	0.42
pOX38-Tc	CS2198 (*rfaJ* Km^R)	90.0	100.0
	CS2187 (*rfaG* Km^R) [d]	0.4	0.4
	CS2193 (*rfaP* Km^R)	0.2	0.2
	CS277 (*ompA* Km^R)	0.42	0.42
R100-1	CS2198 (*rfaJ* Km^R)	21.0	100.0
	CS2187 (*rfaG* Km^R)	23.0	109.0
	CS2193 (*rfaP* Km^R)	26.0	123.0
	CS277 (*ompA* Km^R)	26.0	123.0
ColB2-Tc	CS2198 (*rfaJ* Km^R)	66.7	100.0
	CS2187 (*rfaG* Km^R)	1.6	2.4
	CS2193 (*rfaP* Km^R)	0.89	1.3
	CS277 (*ompA* Km^R)	1.1	1.6

[a] The location of the relevant mutation and the antibiotic resistance marker for each recipient is given in brackets. For a complete description of the genotype and phenotypes see Table 2.4.

[b] A value of 100% was assigned to the wild type strain for each set of experiments. CS2198 was used as the wild type control.

[c] The data represent the average values obtained from four independent experiments.

[d] *rfaG* is polar on *rfaP*.

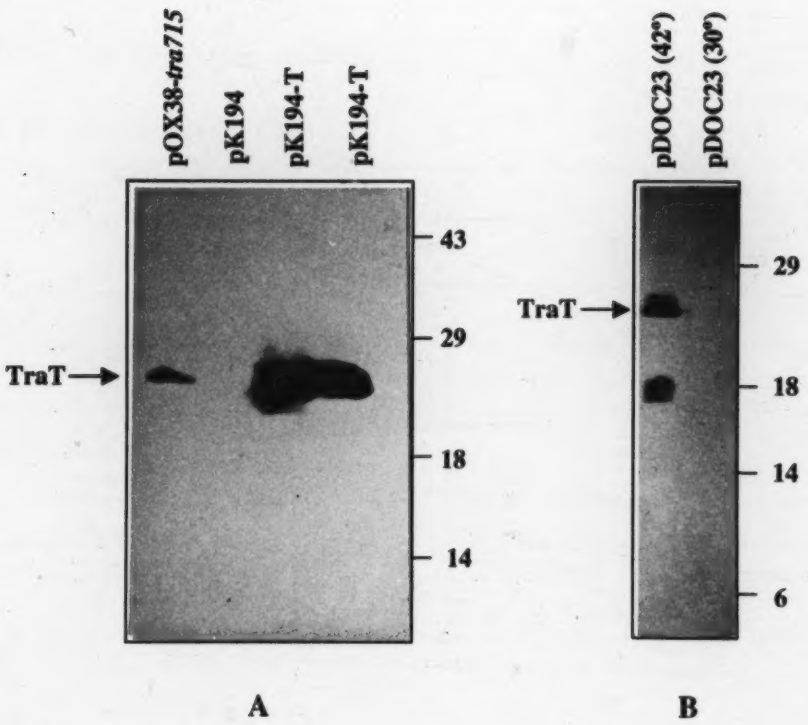
Thus, the sequence differences in the pilin protein do not appear to define the specificity in mating pair formation among F-like plasmids.

2.2.4 Effects of switching pilin genes on surface exclusion specificity

To test if the specificity of TraT-mediated surface exclusion is governed by the pilin sequence, the efficiency of pOX38A::CAT transfer from donor cells expressing the different pilin proteins to recipient cells producing the F TraT protein was examined. In order to achieve constitutive expression of the F TraT protein at high levels, the *traT* gene from the F plasmid was amplified by PCR and was subsequently cloned into the medium copy number vector, pK194 (Section 2.4; Table 2.4). The resulting construct, pK194-T, synthesized the TraT protein constitutively under the *lac* promoter at levels detectable on Western blots as shown in Fig. 2.3A.

Mating experiments were performed with donor *E. coli* XK1200 cells harbouring pOX38A::CAT and either pLF164, pBF203 or pLF107, and *E. coli* JC3272 recipient cells carrying either the vector, pK194 or pK194-T. Control experiments to assay for surface exclusion by cells expressing F TraT from pK194-T, against donor cells carrying either pOX38-Tc, R100-1 or ColB2 plasmids were also carried out simultaneously. The results are summarized in Table 2.3. The surface exclusion index (Sfx index) which is an indicator of the level of inhibition of plasmid transfer, was calculated as the ratio of the mating efficiency obtained with recipients carrying the pK194 vector control versus the mating efficiency to recipients carrying pK194-T as described previously by Harrison *et al.* (1992). The results show that the presence of F TraT in the recipient, as expected, reduced the transfer of pOX38-Tc approximately 30-fold but did not reduce either R100-1 or ColB2 transfer significantly. However, the transfer of pOX38A::CAT was reduced to the same extent with surface exclusion indices greater than 100 when either F, ColB2 or R100-1 pilin was present. In order to confirm the observation that changes in pilin sequence did not affect recognition of TraT, the experiment was repeated with recipient cells expressing the R6-5 TraT protein. The R6-5 plasmid belongs to Sfx IV and as such specifies a surface exclusion system identical to

Fig. 2.3 Western blot analysis of expression of F TraT from pK194-T (A) and R6-5 TraT from pDOC23 (B). Total cellular proteins were separated on a 15% SDS-polyacrylamide gel (Section 4.4). Proteins were transferred to a PVDF membrane for Western blot analysis (Section 4.4). TraT was identified using a polyclonal TraT-specific rabbit antiserum. A. Both pK194 vector control and pK194-T are in *E. coli* JC3272 while the wild type control, pOX38-*tra715*, is in *E. coli* XK100 (Section 4.4). B. *E. coli* LE392 (λ) host cells carrying pDOC23 were grown at 30°C to mid-logarithmic phase then the cells were transfered to 42°C for two hours to induce the production of R6-5 TraT. The position of TraT is marked with an arrow. The molecular mass markers (kDa) are shown on the right.


pOX38-tra715
pK194
pK194-T
pK194-T
TraT
43
29
18
14
A
pDOC23 (42°)
pDOC23 (30°)
TraT
29
18
14
6
B

83

that of R100-1 (Willetts and Maule, 1986). The plasmid, pDOC23 (O'Connor and Timmis, 1987) expresses the R6-5 TraT from a heat-inducible λ P_L promoter. When this plasmid is present in a λ lysogen host carrying the *cI857* mutation, expression of TraT can be induced by inactivation of the λ repressor by growth at 42°C. pDOC23 was transformed into *E. coli* LE392 (λ) cells (Table 2.4). The resulting transformants were initially grown at 30°C then at 42°C to induce the expression of R6-5 TraT. Synthesis of TraT was confirmed by Western blot analysis of total cellular proteins with a TraT-specific polyclonal antiserum. As shown in Fig. 2.3B, TraT is only present in cells grown at 42°C but not at 30°C.

A control mating experiment using donor cells carrying pOX38-Tc, R100-1 or ColB2 revealed that only the transfer of the R100-1 plasmid was reduced in the presence of pDOC23 as previously shown by Harrison *et al.* (1992). Mating assays with donor cells harbouring pOX38A::CAT and either pLF164, pBF203 or pLF107 with recipient LE392 (λ) carrying pDOC23 and pK194, which provides a Km^R marker for convenient selection of transconjugants, were carried out. The experiment was carried out at 30°C and 42°C, which correspond to the uninduced and induced levels of R6-5 TraT expression, respectively. The surface exclusion indices as reported in Table 2.3, were less than 1 in all three cases indicating that the transfer of pOX38A::CAT was not excluded by the presence of R6-5 TraT in the recipient cell. Thus, these results indicate that the pilin sequence is not important for recognition of TraT nor is it important in defining the surface exclusion specificity among F-like plasmids.

2.3 DISCUSSION

In order to understand the role of the pilus in conjugation and f1 phage infection, a genetic analysis of the pilin protein was carried out in this study. Since the pilin genes from F-like plasmids differ from each other with respect to their N-terminal sequences, the importance of the N-terminus of pilin in determining the specificity of pilus function was investigated. The *traA* mutation in pOX38A::CAT was complemented in trans with pilin genes from F, ColB2 and R100-1 plasmids. The transfer efficiency of this plasmid to recipients carrying lesions in the *rfa* or *ompA* loci and to recipients expressing the TraT protein was assayed in order to

Table 2.3 The effect of different alleles of *traA*, encoding pilin, on surface exclusion

Donor strain *E. coli*	Recipient *E. coli*	Transconjugants per 100 donors	Sfx index[a]
XK1200/pOX38-Tc	JC3272/pK194[b]	45.5	1.0
	JC3272/pK194-T[c]	1.4	32.5
XK1200/R100-1	JC3272/pK194	31.4	1.0
	JC3272/pK194-T	18.4	1.7
XK1200/ColB2-Tc	JC3272/pK194	30.0	1.0
	JC3272/pK194-T	5.1	5.9
XK1200/pOX38A::CAT +[d]			
pLF164 (F)	JC3272/pK194	0.65	1.0
	JC3272/pK194-T	0.004	163.0
pLF107 (R100-1)	JC3272/pK194	1.1	1.0
	JC3272/pK194-T	0.006	182.0
pBF203 (ColB2)	JC3272/pK194	0.61	1.0
	JC3272/pK194-T	0.005	113.0
XK1200/pOX38A::CAT +[d]			
pLF164 (F)	LE392/pDOC23 (30°)[e]	0.18	1.0
	LE392/pDOC23 (42°)[f]	1.47	0.12
pLF107 (R100-1)	LE392/pDOC23 (30°)	0.08	1.0
	LE392/pDOC23 (42°)	0.6	0.13
pBF203 (ColB2)	LE392/pDOC23 (30°)	1.0	1.0
	LE392/pDOC23 (42°)	8.8	0.11

[a]The surface exclusion index was calculated as the transfer frequency of the donor plasmid to a recipient not expressing TraT divided by the transfer frequency of the donor plasmid to a recipient expressing TraT as described in Harrison *et al.* (1992) (also see Section 2.4).

[b]pK194 is the vector control.

[c]pK194-T expresses F TraT.

[d]These experiments were repeated at least three times to ensure reproducibility.

[e]pDOC23 at 30° is the uninduced control and is R6-5 TraT.

[f]pDOC23 at 42°C is induced for expression of R6-5 TraT.

determine whether the pilin sequence is important in defining specificity of mating pair formation and surface exclusion. Further, the susceptibility of these cells to f1 and R17 infection was also tested to determine the contribution of pilin sequence to this process.

Analogous to the F plasmid itself, the transfer of pOX38A::CAT to either *rfaP* or *ompA* recipient cells was greatly reduced in the presence of either F, ColB2 or R100-1 pilin. These results indicate that the sequence of pilin does not determine the requirement for PPEA moiety or OmpA for mating pair formation by the F plasmid. Similarly, when either one of the three pilin constructs was present, pOX38A::CAT was excluded to the same extent by recipient cells expressing the F TraT protein. These observations demonstrated that contrary to earlier speculations (Willetts and Maule, 1974), the surface exclusion specificity of the F plasmid is not governed by the pilin sequence. Interestingly, the sequence of the pilin protein also does not determine the susceptibility to pilus-specific phage infection since neither ColB2 nor R100-1 pilin affected the ability of cells harbouring pOX38A::CAT to support f1 and R17 phage growth.

Collectively, the current data with the F transfer system expressing pili belonging to different groups clearly prove that the sequence of the pilin, especially the N-terminus, does not define the specificities noted in mating-pair formation, surface exclusion and f1 infection among F-like plasmids. Willetts (1971) and Ohtsubo *et al.* (1970) reported that when the R100-1 plasmid was co-resident with an F*lac* *traA* mutant, cells carrying these plasmids exhibited markedly reduced sensitivity to both f1 and MS2 (serologically related to R17) phages. This observation is not in agreement with the results obtained in this study and a plausible explanation for this discrepancy could be because their complementation assays employed the whole R100-1 transfer system rather than individual genes. As such, any subtle differences brought about by other pilus assembly proteins would have been overlooked. In this work, with the exception of pilin, all other transfer proteins were supplied by pOX38A::CAT; thus this system permits a finer assessment of the contribution of pilin. Furthermore, the present results are in agreement with those of Finlay *et al.* (1984) who observed that f1 sensitivity was restored to F*lac* *traA* mutants when ColB2 pilin was supplied in trans. R17 phage attachment to pili produced by pOX38A::CAT complemented with

R100-1 pilin was quite unexpected since Frost and Paranchych (1988) had indicated that residues 14-17 in mature pilin are important in phage attachment. This was based on their finding that an F plasmid carrying a Lys to Glu substitution at position 17 in mature pilin, did not attach R17 phages. Residues 15-17 in mature F pilin are also highly conserved in R100-1 pilin but its sequence contains an Asp at position 14 in place of a Thr. The inability of R100-1 pili to attach R17 phages was initially thought to be due to the presence of a charged residue at this position (Frost *et al.*, 1985). If this were true, then the Asp residue is most likely exposed on the surface of R100-1 pili but not the pili synthesized by pOX38A::CAT complemented with R100-1 pilin since the latter attached R17 phages on the sides. These observations suggest a conformational difference between these pili.

Apart from the obvious conclusion on the role of pilin sequence, this study also highlights the possible importance of other pilus assembly proteins in determining the specificity of pilus function. Since the pilus tip is the receptor for f1 phage and it is also involved in recipient cell recognition, the possibility of one or more distinct plasmid-specific pilus tip proteins cannot be ruled out. This possibility could explain the results obtained in this study. Alternatively, the pilin protein might be modified and assembled slightly differently by each F-like plasmid such that the conformation of the pilin subunit in the pilus is different in F-like pili. Of the 15 genes involved in pilus biogenesis, the functions of only three genes *traA, traQ* and *traX* are known (Frost *et al.*, 1994; Moore *et al.*, 1982; 1993). Similar complementation analysis with *traQ* and *traX* mutants with the homologous genes from ColB2 and R100-1 plasmids would be useful in discerning whether the differences in pilin processing and N-terminal acetylation are important in determining the conformation of assembled pilin. In addition, further analysis of pilus assembly proteins is also necessary to pinpoint the hypothetical pilus tip protein.

While pili are important to the transfer process, there is a possibility that they are required only to initiate non-specific contacts with the recipient cell. Specificity in mating pair formation and surface exclusion might be determined at the aggregate stabilization stage when the cells are in wall-to-wall contact. Binding of purified F pili to *ompA* recipients and decreased formation of stable mating aggregates by *ompA* and LPS mutants with F^+ donors,

certainly support this possibility (Achtman *et al.*, 1978a). Since Achtman *et al.* (1977) showed that recipient cells overexpressing the F TraT protein formed fewer mating aggregates with F' donors, TraT might act to block the stabilization process. As such, both TraN and TraG proteins which are required for mating aggregate stabilization, might be important in defining surface exclusion specificity (Manning *et al.*, 1981; Maneewannakul *et al.*, 1992b). At least one domain of TraN has been shown to be exposed on the donor cell surface and as such, TraN might interact with either the LPS or OmpA on recipient cell surface (Maneewannakul *et al.*, 1992b). Further work on F, ColB2 and R100-1 TraN and TraG proteins is needed to define their roles in mating pair formation and surface exclusion.

2.4 MATERIALS and METHODS

2.4.1 Bacterial strains and plasmids

All *E. coli* strains were cultured in liquid Luria Bertani (LB) medium and appropriate antibiotics were added at the following concentrations: ampicillin (Amp) 50 μg/ml; kanamycin (Km) 30 μg/ml; tetracycline (Tc) 20 μg/ml; chloramphenicol (Cm) 25μg/ml; streptomycin (Sm) 200 μg/ml and nalidixic acid (Nal) 40 μg/ml. A complete list of all the bacterial strains and plasmids used in this study is given in Table 2.4.

2.4.2 Bacterial manipulations, recombinant DNA and PCR techniques

Electrocompetent cells for transformation were prepared on a large scale by inoculating 200 ml LB broth with 200 μl of an overnight culture. The culture was grown to mid-exponential phase. The cells were harvested by centrifugation at 2000 g for 10 min at 4°C and the cell pellet was washed twice with 200 ml of ice-cold 0.1 M glucose. The cell pellet was resuspended in 500 μl of 15% glycerol and aliquoted into 50 μl volumes in Eppendorf tubes and frozen by quick-chill in a mixture of ethanol and dry ice prior to storage at -70°C. For electroporation, approximately 10 ng of the DNA devoid of salt was added to the competent cells and the mixture was transferred to a pre-chilled electroporation cuvette and pulsed in a

Table 2.4 Bacterial strains and plasmids used in this study

Strain/Plasmid	Relevant genotype	Reference/Source
Escherichia coli		
CS180-2	*thr leuB6 proA argE his thi galK lacY trpE mtl xyl ara-14 rpsL* Su^+	CS180 reisolated; Austin *et al.* (1990) C. Schnaitman
CS1834	*recBC sbcBC* derivative of JC7623	Pradel and Shnaitman (1991)
CS1999	Δ*lac* of CS180 Mu^R	Klena *et al.* (1992) C. Schnaitman
ED24	F⁻ Lac⁻ Spc^R	Achtman *et al.* (1971)
JC3272	F⁻ *lacΔX74 gal his trp lys rpsL tsx*	Achtman *et al.* (1971)
LE392(λ)	*galT22 hsdR514 lacY1 metB1 supE44 supF58 trpR55 (λ CI857)*	O'Connor and Timmis (1987)
XK1200	F⁻ *lacΔU124* Δ(*nadA aroG gal attλ bio gyrA*)	Moore *et al.* (1981) K. Ippen-Ihler
CS2187	*rfaG13*::Tn*lacZ* of CS1834	C. Schnaitman
CS2193	*rfaP23*::Tn*lacZ* of CS1834	C. Schnaitman
CS2198	*rfaJ*::Tn*lacZ* of CS1999	Pradel *et al.* (1992) C. Schnaitman
MC4100	*araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flb3501 deoC1 ptsF25 rbsR*	C. Manoil
CC102	*galE28* of JC3272	C. Manoil
CC277	*ompA902*::Tn*5* (Km^R) of CC102	Manoil and Rosenbusch (1982) C. Manoil
RD17	F⁻ Δ(*pro lac*)XIII *recA56 rel-1 supE44 thi-1*	Maneewannakul *et al.* (1992a)
Plasmid		
pOX38	Tra^+ $RepFIA^+$	f1 *Hind*III fragment of F; Guyer *et al.* (1980)

Table 2.4 Bacterial strains and plasmids used in this study (cont'd)

pOX38-Km	Tra$^+$ RepFIA$^+$ Km^R	pOX38+*Hind*III fragment of Tn*5* Chandler and Galas (1983)
pOX38-Tc	Tra$^+$ RepFIA$^+$ Tc^R	pOX38::mini Tn*10* Anthony *et al.* (1994)
pOX38A::CAT	Tra$^-$ RepFIA$^+$ *traA*::CAT, Cm^R	Anthony *et al.* (1994)
R100-1	Tra$^+$ Cm^R Fa^R Sm^R Sp^R Su^R Tc^R	Lawn *et al.* (1967); Willetts and Maule (1986)
ColB2-Tc	ColB2 Tra$^+$ Tc^R Cba^R	ColB2::Tn4431, Finlay *et al.* (1984) Anthony *et al.* (1994)
pK194	P15A replicon, Km^R	Jobling and Holmes (1990)
pK194-T	pK194 + F *traT*, Km^R	Anthony *et al.* (1994)
pDOC23	R6-5 *traT* , ColE1 replicon, Ap^R	O'Connor and Timmis (1987)
pLF164	F *traALE* in pUC8, Ap^R	Frost *et al.* (1984)
pLF107	R100-1 *traALE*::IS2 in pUC8, Ap^R	Anthony *et al.* (1994)
pBF203	ColB2 *traALE* in pUC8, Ap^R	Finlay *et al.* (1984)
pUC118-T	F *traT* cloned into *Bam*HI-*Eco*RI of pUC118, Ap^R	This work
pUC8	ColE1 replicon, Ap^R	Vieira and Messing (1982)
pUC118	ColE1 replicon , Ap^R	Vieira and Messing (1987)

Bio-Rad bacterial electro-transformation and pulse controller unit (model 165-2098) using the following parameters: resistance, 200 Ω; capacitance, 25 μF; voltage, 2.5 V. Immediately after, 1 ml LB broth was added and the mixture was transferred to an Eppendorf tube and incubated at 37°C for 60 min. Aliquots were then plated on selective LB agar and the plates were incubated at 37°C overnight for colony development. Preparation of plasmid DNA, restriction endonuclease digestions and ligations were performed using standard procedures (Sambrook *et al.*, 1989). DNA fragments to be ligated were routinely purified from 0.7% agarose gels using silanized glass wool following gel electrophoresis (Sambrook *et al.*, 1989). PCR was carried out using Vent DNA polymerase (New England Biolabs), 100 ng of template DNA and 50 pmoles of the appropriate oligonucleotide primers. For amplification of fragments smaller than 3 kb, the reaction was carried out for 30 cycles in which each cycle consisted of (i) denaturation at 93°C for 0.5 min (ii) annealing at 50°C for 0.5 min (iii) elongation at 72°C for 1 min.

2.4.3 Qualitative phage spot tests

Phage sensitivity was determined using a modified procedure described in Frost and Paranchych (1988). Approximately $1x10^7$ cells of the bacteria to be tested were mixed with 3 ml of 0.7% LB top agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 7 g agar/ 1 l H_2O; Ausubel *et al.*, 1989) and used as an overlay lawn on LB agar plates containing appropriate antibiotics. Both f1 and R17 phage suspensions containing approximately $1x10^{11}$ pfu/ml were serially diluted in PBS pH 7.3 (8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4.7H_2O$, 0.2 g KH_2PO_4; Ausubel *et al.*, 1989) and 10 μl of the various dilutions (ranging from $1x10^9$ to $1x10^1$ pfu) was spotted onto the lawn. After incubation at 37°C overnight, lysis at various dilutions were observed and the efficiency of plating was scored as follows: (+++) fully sensitive, lysis observed with $1x10^2$ pfu; (++) moderately sensitive, lysis observed with $1x10^4$ pfu; (+) weakly sensitive, lysis observed with $1x10^8$ pfu; (R) resistant, no lysis.

2.4.4 Transmission electron microscopy (TEM)

Sample preparation for electron microscopy was essentially as described by Maher *et al.*(1993). Phages, f1 and R17, were mixed with approximately $1x10^7$ cells at multiplicity of infection (MOI) of 10 and 100, respectively. The phages were allowed to adsorb to pili on ice for 30 min. Ten µl of the phage/bacteria mixture was transferred onto a freshly prepared Formvar-coated, carbon- coated copper grid using a wide-bore pipette tip. The sample was allowed to adsorb for 0.5 min and the unadsorbed material was removed by blotting the edge of the grid with a Whatman filter paper. The samples were negatively stained with 1% phosphotungstic acid pH 7.0 for 10 sec and examined with a Philips model 410 transmission electron microscope.

2.4.5 Mating assays and determination of mating efficiency profiles

Donor and recipient bacteria were grown in LB broth at either 37°C or 30°C to mid-logarithmic phase (OD_{600} of 0.4-0.6). 100 µl each of the donor and recipient cell cultures were mixed in 1 ml fresh LB broth and the mixture was incubated at the appropriate temperature for 60 min. The mating was interrupted by vortexing vigorously and the mixture was chilled on ice. Dilutions were made in ice-cold 1xSSC buffer, pH 7.0 (0.15 M NaCl, 0.015 M $Na_3C_6H_5O_7.2H_2O$; Frost *et al.*, 1984) and 10 µl of each dilution was spotted on LB agar selective for either the donors or the transconjugants. The efficiency of mating was recorded as the number of transconjugants per 100 donor cells and the overall proficiency of mating relative to a wild type control was expressed as the percent Control as reported earlier (Anthony *et al.*, 1994).

2.4.6 Construction of pOX38A::CAT

The construction of pOX38A::CAT took advantage of a unique *Nae*I site at the junction between the leader sequence and the coding sequence of the pilin gene, *traA* (Frost *et al.*, 1985). A 792-bp chloramphenicol acetyltransferase GenBlock (CAT, Pharmacia LKB Biotechnology) was digested with *Hind*III restriction endonuclease and the ends were converted to blunt ends by Klenow fragment I. The blunt-ended fragment was subsequently

ligated into the *Nae*I site of *traA* in the plasmid, pLF164. The ligation mixture was electroporated into competent DH5α cells. Transformants were initially selected for chloramphenicol resistance then the Cm^R colonies were selected for ampicillin resistance by replica plating. Plasmid DNA from Amp^R and Cm^R colonies was digested with *Eco*RV and *Sca*I to confirm the insertion and to determine the orientation of the CAT cassette with respect to the *traA* gene. The selected construct, pLF164A::CAT which contained the CAT cassette inserted in the direction of *traA* transcription as confirmed by DNA sequencing (Section 3.4) was used in the following procedure to cross this mutation onto the pOX38 plasmid.

A schematic of the procedure used to create a pOX38 derivative carrying an interrupted *traA* gene is illustrated in Fig. 2.2B. Triparental matings (Kathir and Ippen-Ihler, 1991; Moore *et al*, 1987) were performed by mixing 100 µl each of the donor strain, *E. coli* RD17 harbouring pOX38 (Table 2.4), with a *rec*$^+$ *E. coli* JC3272 recipient strain carrying pLF164A::CAT. Matings were performed as described earlier. After 1 hour, a second recipient, *E. coli* XK1200 (Nal^R), was added to the mating mixture in four-fold excess and incubation was continued for a further 6 hours. After allowing *in vivo* recombination to occur, transconjugants were selected for chloramphenicol and nalidixic acid resistance and and screened for ampicillin sensitivity. The resulting plasmid, pOX38A::CAT, was tested for the presence of the CAT insert by restriction analysis. In addition, the *traA* gene in pOX38A::CAT was amplified by PCR using pOX38A::CAT as the template and two primers flanking the pilin gene, KAN-A2 (5'-CGGAATTCCTGTCAGAGGCCAACGA-3') and KAN-A3 (5'-AGATGAGGCTGCAGACAGAG-3'). A product, approximately 1.3 kb which was 800 bp larger than wild type *traA*, confirmed the presence of the CAT cassette.

2.4.7 Construction of pK194-T

In order to achieve high levels of expression of the F TraT protein, the *traT* gene was amplified by PCR using pOX38-Km as the template and two oligonucleotide primers, KAN-T1 (5'-CGGGATCCCGGCGCAATTTTAGTGACGG-3') and KAN-T2 (5'-CGGAATTCCGCCGGCAACACTTCACG-3') derived from sequences bordering the F *traT*

including its promoter and the inverted repeats at either end of the gene (Jalajakumari *et al.*, 1987 and Frost *et al.*, 1994). The 1057 bp amplifed fragment was initially cloned into pUC118 vector in the opposite direction to the *lac* promoter (Vieira and Messing, 1987) using the *Bam*H1 and *Eco*R1 restriction sites incorporated into KAN-T1 and KAN-T2, respectively. The DNA sequence of the *traT* gene in the resulting construct, pUC118-T, was determined by DNA sequencing (Section 3.4) in order to detect any PCR-induced mutations. The *Bam*H1 and *Eco*R1 fragment from pUC118-T was subsequently cloned into the medium copy number vector, pK194 (Jobling and Holmes, 1990), to give rise to pK194-T. This plasmid expressed TraT constitutively from the *lac* promoter. Expression of TraT in *E. coli* JC3272 cells harbouring pK194-T was confirmed by Western blot analysis (Section 4.4) of total cellular proteins with a polyclonal TraT-specific rabbit antiserum.

2.4.8 Surface Exclusion assays

Surface exclusion assays were performed in an identical manner to mating assays except the recipient, *E. coli* JC3272, carried either the pK194 vector or expressed F TraT constitutively from pK194-T. For performing surface exclusion assays with recipient cells expressing R6-5 TraT, 2 ml cultures of *E. coli* LE392 (λ) cells carrying the plasmids, pDOC23 and pK194 were grown at 30°C for 3 hours. The vector, pK194, provided a convenient kanamycin resistance marker for selection of transconjugants. One millilitre aliquot of the culture was grown at 42°C for 2 hours to induce the expression of R6-5 TraT from the λ P_L promoter (Harrison *et al.*, 1992). Matings were carried out with *E. coli* XK1200 harbouring pOX38A::CAT and one of either pLF164, pBF203 or pLF100 as donors. Either uninduced or induced *E. coli* LE392 (λ) carrying pDOC23 and pK194 were used as the recipients. Matings with induced recipients were carried out at 42°C while matings with uninduced recipients were done at 30°C. Transconjugants were assayed for ampicillin, chloramphenicol and kanamycin resistance and donors were selected for ampicillin, chloramphenicol and nalidixic acid resistance. The surface exclusion index was calculated as the mating efficiency obtained with a recipient not expressing TraT divided by the mating efficiency with a recipient expressing TraT as described by Harrison *et al.* (1992).

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Willetts, N.S. and Maule, J. (1986) Specificities of IncF plasmid conjugation genes. Genet. Res. **47**:1-11.

CHAPTER 3

Analysis of the *traLEKBP* sequence and the TraP protein from three F-like plasmids: F, R100-1 and ColB2*

*A version of this chapter has been published. Anthony, K.G., Kathir, P., Moore, D., Ippen-Ihler, K., and Frost, L.S. (1996) J. Bacteriol. 178:3194-3200. The work described in this chapter was solely carried out by the first author.

3.1 INTRODUCTION

Electron microscope heteroduplex mapping studies have shown that the *tra* region of F-like plasmids are highly homologous (Sharp *et al.*, 1973). However, differences in protein sequence among gene products involved in conjugative DNA metabolism have been found to be clustered in small domains. These regions of non-homology have been correlated with plasmid-specificity (Frost *et al.*, 1994).

The present study was undertaken in an attempt to determine variation in protein sequence among gene products involved in pilus synthesis and to assess a correlation between sequence differences with pilus specificity. In this chapter, the nucleotide sequences of *traK*, *B* and *P* genes from the R100-1 plasmid were determined. Further, the DNA sequences of *traL*, *E*, *K*, *B*, and *P* genes from both ColB2 and R100-1 plasmids were compiled and their gene products were compared with those of F to determine the extent of sequence variation. While a high degree of sequence conservation was detected among TraL, E, K and B proteins, the gene product of *traP* showed significant sequence variation.

Although TraL, E, K and B are essential for piliation, mutations affecting *traP* reduce DNA transfer. A derivative of pOX38 with a kanamycin resistance cassette inserted in the *traP* gene, pOX38-*traP474*, exhibited reduced levels of DNA transfer and sensitivity to pilus-specific phages but produced pili that were longer than normal (Anthony *et al.*, 1996). Such characteristics might be expected of a mutation in a pilus tip protein which might affect pilus length determination, recipient cell recognition and f1 phage attachment. In light of the sequence differences in the three TraP proteins, TraP seemed to be a promising candidate for a pilus tip adhesin. To investigate this possibility, the pOX38-*traP474* mutation was complemented with chimeric plasmids expressing the *traP* alleles from ColB2 and R100-1 plasmids and the resulting phenotype was assayed for susceptibility to pilus-specific phage infection. Further, mating assays with recipient cells carrying mutations in the *rfa* loci allowed an assessment of the role of TraP in recognition of LPS receptors. In addition, the topology of TraP in the inner membrane was analyzed by Tn*phoA* mutagenesis.

3.2 RESULTS

3.2.1 Cloning, determination and analysis of nucleotide sequences of the R100-1 plasmid *traK, B* and *P* genes

A 3 kb fragment spanning the distal ends of *traE* and *trbD* of R100-1 (corresponding to coordinates 3437-6424 in F) containing *traK, B* and *P* genes was amplified by PCR. This fragment was subsequently cloned into the *Bam*HI and *Eco*RI sites of pUC118 vector (Vieira and Messing, 1987) to create the plasmid, pKAR-2 (Table 3.4). Another chimera, pKAR-P1, which contains the R100-1 *traP* gene (corresponding to F coordinates 5625-6424) cloned in isolation into the *Hin*dIII-*Eco*RI sites of pUC118, was also constructed in a similar manner.

The nucleotide sequences of *traK, B* and *P* genes encoded by these chimeras were determined as described in Section 3.4. The entire DNA sequence is presented in Fig. 3.1. Three complete open reading frames (ORF) corresponding to *traK*, *traB* and *traP* and a partial ORF for *trbD* are shown along with the deduced ATG codons preceded by Shine-Dalgarno consensus sequences. Translation initiating at these codons would give rise to 243, 486 and 195 amino acid products with calculated molecular weights of 25 627, 51 872 and 21 861 for *traK*, *traB* and *traP*, respectively. Closely spaced or overlapping start and stop codons of adjacent cistrons often seen in the F transfer operon (Frost *et al.*, 1994), are also conserved in this region of the R100-1 sequence. The *traK* stop codon overlaps with the start codon of *traB*. While the *traB* stop codon is positioned in the *traP* coding region, the *traP* start is at the 3' end of the *traB* coding sequence. A potential intragenic rho-dependent terminator or *rut* sequence which is present in the F *traK* cistron (nucleotides 3966-3971, Frost *et al.*, 1994; Penfold *et al.*, 1994), is also present in the R100-1 sequence at nucleotides 514-519 as shown in Fig. 3.1. The restriction enzyme sites shown also occur in the F sequence. This region of the R100-1 sequence shows overall conservation with the F transfer operon at the level of gene organization as well as nucleotide sequence.

Fig. 3.1 Nucleotide sequence of R100-1 *traKBP* region. The sequence of the distal ends of *traE* to *trbD* (F coordinates 3437-6424; Frost *et al.*, 1994) is shown. Sequences corresponding to the ribosome-binding site consensus are underlined and stop codons are indicated by asterisks. ATG initiation codons are enclosed in grey boxes and the arrows indicate the direction of the transcript. The coordinates for the beginning and the end of each ORF are shown in brackets adjacent to the arrow. The potential rho-dependent termination sequence in *traK* is enclosed in a grey box with dotted outline. Recognition sequences for restriction endonucleases which are also present in the F sequence are shown.

traK (63-791)
PstI
PvuII
rho-dependent termination
PstI
traB (841-2301)
traK stop
SphI
SmaI

```
1351  GTCTGTGCCGGTGCCTAACCGGATACAGCGTAAGGTGTTTACCCGTAATG  1400

1401  AGGGAAAACAGGGACCATCGCTGCCGTACATTCCGTCAGGAAGTTTTGCG  1450

1451  AAAGCCATGCTGATTGAAGGGGCGGATGCCAATGCCTCAGTCACCGGTAA  1500

1501  TGAATCCACGGTGCCGATGCAGTTGCGTATCACCGGCCTGGTGGAAATGC  1550

1551  CGAACAGCAAGACGTATGACGCAACGGGATGTTTTGTGGGGCTGGAAGCC  1600

1601  TGGGGGGATGTGTCCAGTGAACGTGCCATTGTACGCACCCGCAATATCAG  1650

1651  TTGCCTGAAGGACGGCAAAACTATTGATATGCCGATTAAGGGGCATGTCA  1700

1701  GCTTCCGGGGTAAAAACGGTATCAAGGGCGAAGTGGTGATGCGTAACGGC  1750

1751  AAAATCCTCGGCTGGGCATGGGGCGCGGGATTTGTTGACGGTATCGGTCA  1800

1801  GGGAATGGAGCGCGCCTCCCAACCGGCTGTCGGGCTGGGTGCCACAGCCG  1850

1851  CTTACGGGGCTGGTGATGTCCTGAAAATGGGTATCGGTGGCGGCGCATCG  1900

1901  AAAGCCGCACAGACGCTCAGTGACTACTACATCAAACGTGCCGAACAGTA  1950

1951  TCACCCGGTGATACCGATTGGTGCGGGCAACGAAGTGACCGTGGTGTTCC  2000

2001  AGGACGGCTTCCAGCTGAAAACCGTGGAGGAAATGGCACTGGAGCAGTCA  2050
                PvuII

2051  CAGAGTACAGCGGAAGAGGGTAACCCGGAAAGCCCGGTGCCGAATCCTCC  2100

2101  CTCGGCAGAAAGCCACCTGAACGGTTTTAACACCGACCAGATGCTGAAAC  2150

2151  AGCTGGGCAATCTGAATCCGCAGCAGTTTATGTCCGGAGCACAAGAACCC  2200
      PvuII                                  traP (2283-2849)

2201  GCTGGGCGGGGATGCCGGCAAGGAGGGCGGAAATGGCGGGTGATGCACTG  2250
                                          traB stop  *

2251  ACATTCCGTGTGGCGTTTGTCGTTCGCTGGCTGCTGTGGGTCGTCAGGTT  2300

2301  TGTGGTTATCTGGCCGCTGGCCACAATGGCCCTGATGGCTCTCTTTGTTT  2350

2351  TATGGAAGGACAACACCACGCCGGGGAAATTACTGGTTAAGGAGATTAAC  2400

2401  TTCGTAAGACAAACAGCACCTGCCGGACAGTTCCCGGTCAGTGAATGCTG  2450

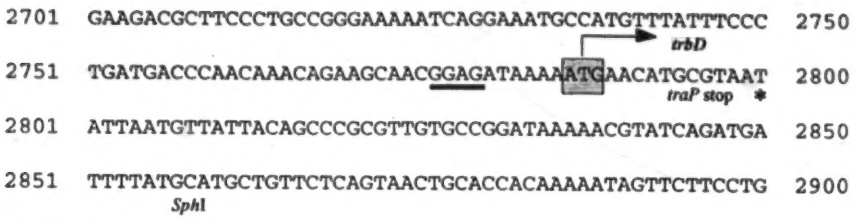
2451  GTTTTCTTCCTCTGATTCTTCAGGCAGATCAGAAATACAGGATATCTGCC  2500

2501  ATTACCGCGCGGCTGATGCTGCAGATTATGTCAGGGAGACTGACCGGTCA  2550
                      PstI

2551  CTGATGCAACTGGTCACCGCATTCTGGCCAACCCTGGCACTGATGTATAT  2600

2601  GTCGTTTGCGGCAGGCACCGGGAAATATCCGGTGCTGCCCGGAAAAATGA  2650

2651  AATGTATCCGGGTGGTAACCGCAGATGAATATCTGAAGGACGTTTATACG  2700
```


2701 GAAGACGCTTCCCTGCCGGGAAAAATCAGGAAATGCCATGTTTATTTCCC 2750
trbD
2751 TGATGACCCAACAAACAGAAGCAACGGAGATAAAAATGAACATGCGTAAT 2800
traP stop *
2801 ATTAATGTTATTACAGCCCGCGTTGTGCCGGATAAAAACGTATCAGATGA 2850
2851 TTTTATGCATGCTGTTCTCAGTAACTGCACCACAAAAATAGTTCTTCCTG 2900
SphI
2951 CACCG 2955

3.2.1 Comparative analyses of TraL, E, K, B and P proteins from F, ColB2 and R100-1 plasmids

The nucleotide sequences of the *tra* region encompassing *traL, E, K, B* and *P* from F and ColB2 plasmids and the *traL* and *E* region from R100-1 have been previously determined (Frost *et al.*, 1994 and Anthony *et al.*, 1996). The amino acid sequences of all five proteins from the three plasmids were compiled and analyzed using the multiple sequence alignment program (Section 3.4) in order detect the degree of conservation at the protein level. The results are presented in Fig. 3.2a, b, c, d and e.

TraL and TraE

Based on the sequence comparison shown in Fig. 3.2a, the ColB2 TraL protein shows 100% identity with its F counterpart while the R100-1 TraL protein has a single Ile to Val transition at position 38. The *traL* gene which is located immediately downstream of the pilin gene, *traA*, encodes a 91 aa polypeptide predicted to be a peripheral membrane protein with a domain for membrane association extending from aa 31-61 (Frost *et al.*, 1994). Although TraL is essential for piliation, it has never been visualized in protein labelling experiments in maxicells, suggesting that its expression is highly regulated (Frost *et al.*, 1994). Sequence homology among TraL, TrbD of the broad-host range IncPα plasmid, RP4, and the VirB3 protein of the Ti plasmid of *Agrobacterium tumefaciens* all of which are required for DNA transfer, has been documented (Lessl *et al.*, 1992; Kado, 1994).

The *traE* gene product is a 188-aa inner membrane protein required for pilus assembly. It exhibits a predominantly hydrophilic character and has a single predicted transmembrane domain (aa 12-33) near its N-terminus (Frost *et al.*, 1994). Achtman *et al.* (1979) showed that TraE is associated with the inner membrane and, as such, this protein is most likely anchored in the inner membrane via its N-terminus with the remainder of the protein in the periplasm. Comparison of TraE sequences shown in Fig. 3.2b reveals that the R100-1 sequence has several single aa substitutions throughout the central region of the molecule. These changes are: $T_{47}\rightarrow P$, $A_{53}\rightarrow G$, $A_{56}\rightarrow V$,$S_{86}\rightarrow P$, $T_{88}\rightarrow I$, $R_{118}\rightarrow L$, $N_{121}\rightarrow K$, $A_{140}\rightarrow V$, $V_{147}\rightarrow I$,

Fig. 3.2 Multiple sequence alignment of (a) *traL*, (b) *traE*, (c) *traK*, (d) *traB* and (e) *traP* gene products from F, ColB2 and R100-1 plasmids. The alignments were generated with the program PILEUP (GCG package; Devereux *et al.*, 1984) using the Dayhoff 250 PAM matrix (Dayhoff *et al.*, 1978), a gap-weight of 3.0 and a gap-length-weight of 0.2. The three sequences are shown in the order F, ColB2 and R100-1. Dashes indicate gaps introduced for alignment. Regions of non-homology are enclosed in grey boxes. The two putative transmembrane segments (TMS I & II) identified in TraP, are indicated by horizontal bars above the F sequence (Fig. 3.2e). The triangles (Fig. 3.2e) mark the precise locations of Tn*phoA* insertions (refer to Fig. 3.3).

Fig.3.2a TraL

```
F (L)       MSGDENKLKKYRFPETLTNQSRWFGLPLDELIPAAICLGWGITTSKYLFG 50
ColB2 (L)   MSGDENKLKKYRFPETLTNQSRWFGLPLDELIPAAICLGWGITTSKYLFG 50
R100-1 (L)  MSGDENKLKKYRFPETLTNQSRWFGLPLDELIPAAICLGWGITTSKYLFG 50

            IGAAVLVYFGIKKLKKGRGSSWLRDLIYWYMPTALLRGIFS 92
            IGAAVLVYFGIKKLKKGRGSSWLRDLIYWYMPTALLRGIFS 92
            IGAAVLVYFGIKKLKKGRGSSWLRDLIYWYMPTALLRGIFS 92
```

Fig. 3.2b TraE

```
F (E)       MEHGARLSTSRVMAIAFIFMSVLIVLSLSVNVIQGVNNYRLQNEQRAVT 50
ColB2 (E)   MEHGARLSTSRVMAIAFIFMSVLIVLSLSVNVIQGVNNYRLQNEQRAVT 50
R100-1 (E)  MEHGARLSTSRVMAIAFIFMSVLIVLSLSVNVIQGVNNYRLQNEQRAVT 50

            PMAFNAPFAVSQNSADASYLQQMALSFIALRLNVSSETVDASHQALLQYI 100
            PMAFNAPFAVSQNSADASYLQQMALSFIALRLNVSSETVDASHQALLQYI 100
            PMAFNAPFAVSQNSADASYLQQMALSFIALRLNVSSETVDASHQALLQYI 100

            RPGAQNQMKVILAEEAKSIKSDNVNSAFFQTSVRVCLSMAVWKFRGVDKT 150
            RPGAQNQMKVILAEEAKSIKSDNVNSAFFQTSVRVCLSMAVWKFRGVDKT 150
            RPGAQNQMKVILAEEAKSIKSDNVNSAFFQTSVRVCLSMAVWKFRGVDKT 150

            WIGNSKPFTDIKHYILILKRENGVTWLDNFGETDDEKK 189
            WIGNSKPFTDIKHYILILKRENGVTWLDNFGETDDEKK 189
            WIGNSKPFTDIKHYILILKRENGVTWLDNFGETDDEKK 189
```

Fig. 3.2c TraK

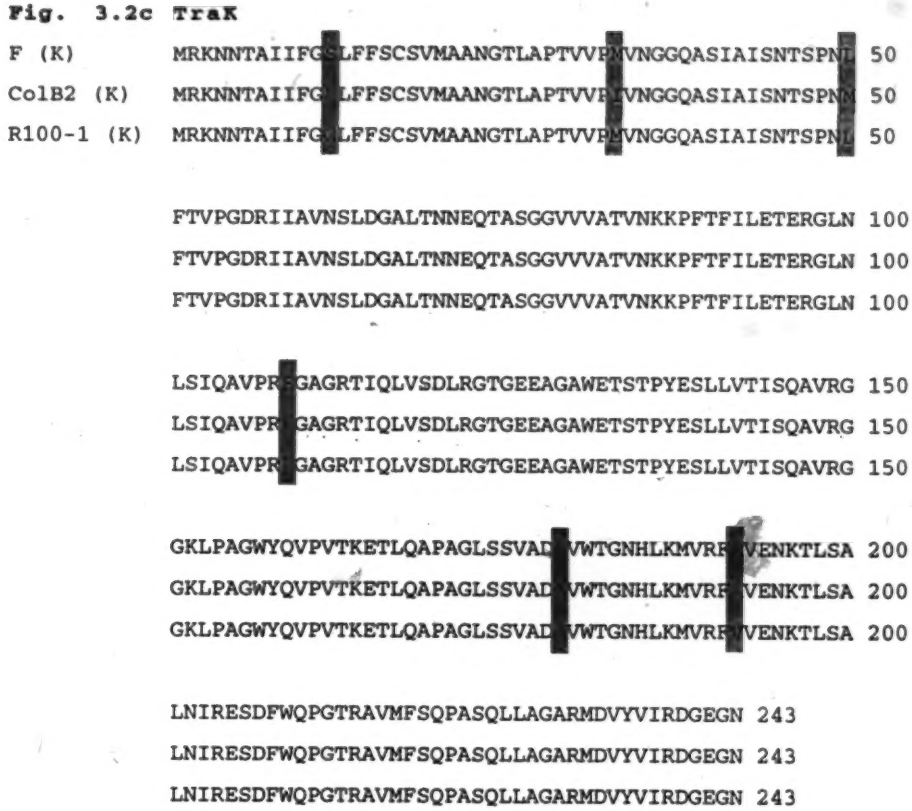

F (K) MRKNNTAIIFGLFFSCSVMAANGTLAPTVVPMVNGGQASIAISNTSPNL 50
ColB2 (K) MRKNNTAIIFGLFFSCSVMAANGTLAPTVVPIVNGGQASIAISNTSPNN 50
R100-1 (K) MRKNNTAIIFGLFFSCSVMAANGTLAPTVVPMVNGGQASIAISNTSPNL 50
FTVPGDRIIAVNSLDGALTNNEQTASGGVVVATVNKKPFTFILETERGLN 100
FTVPGDRIIAVNSLDGALTNNEQTASGGVVVATVNKKPFTFILETERGLN 100
FTVPGDRIIAVNSLDGALTNNEQTASGGVVVATVNKKPFTFILETERGLN 100
LSIQAVPRGAGRTIQLVSDLRGTGEEAGAWETSTPYESLLVTISQAVRG 150
LSIQAVPRGAGRTIQLVSDLRGTGEEAGAWETSTPYESLLVTISQAVRG 150
LSIQAVPRGAGRTIQLVSDLRGTGEEAGAWETSTPYESLLVTISQAVRG 150
GKLPAGWYQVPVTKETLQAPAGLSSVADVWTGNHLKMVRPVENKTLSA 200
GKLPAGWYQVPVTKETLQAPAGLSSVADVWTGNHLKMVRPVENKTLSA 200
GKLPAGWYQVPVTKETLQAPAGLSSVADVWTGNHLKMVRPVENKTLSA 200
LNIRESDFWQPGTRAVMFSQPASQLLAGARMDVYVIRDGEGN 243
LNIRESDFWQPGTRAVMFSQPASQLLAGARMDVYVIRDGEGN 243
LNIRESDFWQPGTRAVMFSQPASQLLAGARMDVYVIRDGEGN 243

Fig. 3.2d TraB

```
F (B)        MASINTIVKRKQYLWLGIVVVGTASAIGGALYLSDVDMSGNGETVAEQEP 50
ColB2 (B)    MASINTIVKRKQYLWLGIVVVGTASAIGGALYLSDVDMSGNGETVAEQE? 50
R100-1 (B)   MASINTIVKRKQYLWLGIVVVGTASAIGGALYLSDVDMSGNGETVAEQEP 50

             VPDMTGVVDTTFDDKVRQHATTEMQVTAAQMQKQYEEIRRELDVLNKQRG 100
             VPDMTGVVDTTFDDKVRQHATTEMQVTAAQMQKQYEEIRRELDVLNKQRG 100
             VPDMTGVVDTTFDDKVRQHATTEMQVTAAQMQKQYEEIRRELDVLNKQRG 100

             DDQRRIEKLGQDNAALAEQVKALGANPVTATGEPVPQMPAPPGPEGEPQ 150
             DDQRRIEKLGQDNAALAEQVKALGANPVTATGEPVPQMPAPPGPEGEPQ 150
             DDQRRIEKLGQDNAALAEQVKALGANPVTATGEPVPQMPAPPGPEGEPQ 150

             PGNTPVSFPPQGSVAVPPPTAFYPGNGVTPPPQVTYQSVPVPNRIQRKVF 200
             PGNTPVSFPPQGSVAVPPPTAFYPGNGVTPPPQVTYQSVPVPNRIQRKVF 200
             PGNTPVSFPPQGSVAVPPPTAFYPGNGVTPPPQVTYQSVPVPNRIQRKVF 200

             TRNEGKQGPSLPYIPSGSFAKAMLIEGADANASVTGNESTVPMQLRITGL 250
             TRNEGKQGPSLPYIPSGSFAKAMLIEGADANASVTGNESTVPMQLRITGL 250
             TRNEGKQGPSLPYIPSGSFAKAMLIEGADANASVTGNESTVPMQLRITGL 250

             VEMPNSKTYDATGCFVGLEAWGDVSSERAIVRTRNISCLKDGKTIDMPIK 300
             VEMPNSKTYDATGCFVGLEAWGDVSSERAIVRTRNISCLKDGKTIDMPIK 300
             VEMPNSKTYDATGCFVGLEAWGDVSSERAIVRTRNISCLKDGKTIDMPIK 300

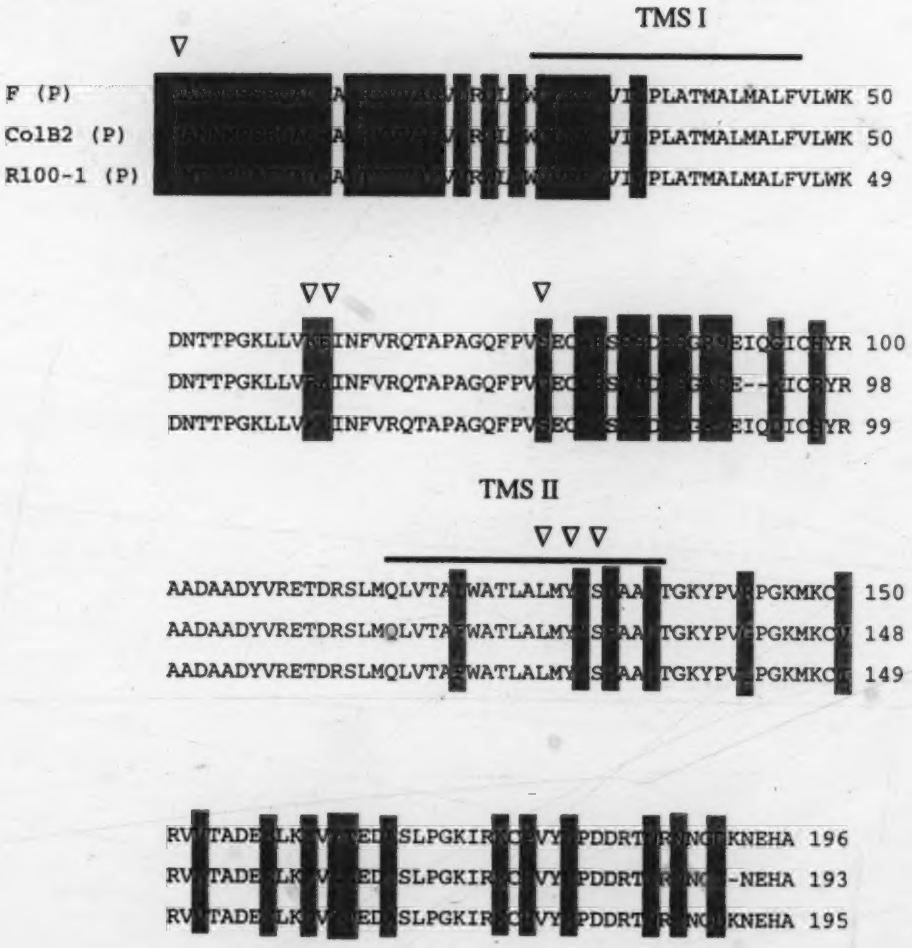
             GHVSFRGKNGIKGEVVMRNGKILGWAWGAGFVDGIGQGMERASQPAVGLG 350
             GHVSFRGKNGIKGEVVMRNGKILGWAWGAGFVDGIGQGMERASQPAVGLG 350
             GHVSFRGKNGIKGEVVMRNGKILGWAWGAGFVDGIGQGMERASQPAVGLG 350

             ATAAYGAGDVLKMGIGGGASKAAQTLSDYYIKRAEQYHPVIPIGAGNEVT 400
             ATAAYGAGDVLKMGIGGGASKAAQTLSDYYIKRAEQYHPVIPIGAGNEVT 400
             ATAAYGAGDVLKMGIGGGASKAAQTLSDYYIKRAEQYHPVIPIGAGNEVT 400

             VVFQDGFQLKTVEEMALE[redacted]NPESPVP[redacted]PPSAESHLNGFNTDQ 450
             VVFQDGFQLKTVEEMALE[redacted]NPESPVP[redacted]PPSAESHLNGFNTDQ 450
             VVFQDGFQLKTVEEMALE[redacted]NPESPVP[redacted]PPSAESHLNGFNTDQ 450
```

MLKQLGNLNPQQFMSG
MLKQLGNLNPQQFMSG
MLKQLGNLNPQQFMSG
475
475
486

Fig. 3.2e TraP


TMS I
F (P)
ColB2 (P)
R100-1 (P)
PLATMALMALFVLWK 50
PLATMALMALFVLWK 50
PLATMALMALFVLWK 49
DNTTPGKLLVNEINFVRQTAPAGQFPVSEC
DNTTPGKLLVNEINFVRQTAPAGQFPVSEC
DNTTPGKLLVNEINFVRQTAPAGQFPVSEC
EIQGICHYR 100
EIQGICRYR 98
EIQGICHYR 99
TMS II
AADAADYVRETDRSLMQLVTAFWATLALMY
AADAADYVRETDRSLMQLVTAFWATLALMY
AADAADYVRETDRSLMQLVTAFWATLALMY
TGKYPVRPGKMKC 150
TGKYPVGPGKMKC 148
TGKYPVRPGKMKC 149
SLPGKIRKC
SLPGKIRKC
SLPGKIRKC
KNEHA 196
-NEHA 193
KNEHA 195

L_{148}→R, D_{154}→N, K_{156}→E. The TraE proteins of F and ColB2, however, are identical. Sequence similarity among TraE, TrbF of RP4 and VirB5 of the Ti plasmid has been identified (Lessl *et al.*, 1992; Kado, 1994).

TraK

Analysis of F and ColB2 TraK proteins revealed amino acid substitutions at three positions namely, M_{33}→I, L_{50}→M and E_{109}→K (Fig. 2c). Likewise, the R100-1 TraK protein also contains changes at three positions specifically, S_{12}→G, A_{179}→S and A_{192}→V (Fig. 3.2c). Apart from these changes, the sequence of the remainder of the 242 aa polypeptide including the signal sequence is remarkably conserved in all three plasmids. Expression of *traK* is essential for piliation and its product is predicted to be located in the periplasm. The 24 kDa mature form of TraK arising after the cleavage of the signal sequence has been visualized previously by [^{35}S]-methionine-labelling of λ transducing phages carrying the *traB-C* region (Moore *et al.*, 1987).

TraB

The F *traB* gene encodes a 475 aa polypeptide. The 50.5 kDa mature product is predicted to be predominantly hydrophilic and as such is believed to be positioned in the periplasm and anchored in the inner membrane via a short transmembrane segment at its N-terminus (Frost *et al.*, 1994). A 60 kDa protein corresponding to TraB has been visualized on SDS-polyacrylamide gels (Moore *et al.*, 1987). Amino acid sequence comparisons presented in Fig. 3.2d reveal that the TraB protein of ColB2 also contains 475 aa and has 100% sequence identity to F TraB. The polypeptide specified by R100-1 *traB* is 486 aa long with 11 additional amino acids at the C-terminus (Fig. 3.2d). In addition to the C-terminal extension, the following amino acid substitutions occur in the R100-1 sequence: S_{140}→P, R_{419}→Q, T_{420}→S, R_{423}→T, D_{427}→G, V_{435}→N, S_{467}→A, G_{469}→E, G470→P, G_{471}→A, N_{472}→G, D_{473}→R, K_{475}→C (Fig. 3.2d). The high proportion of proline (8%) and glycine (11.7%) residues, a feature notable in the F TraB sequence, is also conserved in ColB2 and R100-1 TraB proteins.

Sequence homology among TraB, TrbI and VirB10 proteins of RP4 and Ti plasmids, respectively have been documented (Lessl *et al.*, 1992; Kado, 1994).

TraP

As shown in Fig. 3.2e, the F *traP* gene encodes a polypeptide of 196 residues while ColB2 and R100-1 *traP* genes specify products of 192 and 195 amino acids, respectively. Using λ transducing phages carrying the F *traP* gene, a 21.5 kDa polypeptide has been identified as the gene product of *traP* (Moore *et al.*, 1987). Sequence comparisons of the three proteins reveal short defined regions of non-homology among the three plasmids. The N-terminal residues of R100-1 TraP significantly differ from those of F and ColB2. Major changes in ColB2 TraP, however, occur in a stretch of amino acids at positions 81-96 with several single substitutions positioned throughout the distal end of the protein (Fig. 3.2e). Secondary structure predictions and hydropathy analysis revealed TraP as an integral membrane protein with two transmembrane segments at residues 25-49 and 118-138 indicated as overlined segments in Fig. 3.2e.

GenBank nucleotide sequence accession numbers

The DNA sequence of R100-1 *traL-P* and ColB2 *traL-P* can be obtained from GenBank database using the accession numbers U51859 and U51860, respectively.

3.2.3 Determining the membrane topology of TraP by Tn*phoA* mutagenesis

In order to gain more insights into the nature of TraP and to identify the position of the variable region in the molecule, the orientation of TraP in the inner membrane was investigated by Tn*phoA* insertion mutagenesis. The λTn*phoA* delivery system (Manoil, 1991) which generates gene fusions to the *E. coli phoA* gene encoding alkaline phosphatase (AP) was used for this purpose. This system is a convenient tool to analyze the topology of membrane proteins since only fusions which position AP in the periplasm give rise to enzymatic activity. In order to obtain specific transpositions in the *traP* gene, pKI250 which carries the F *traP-V* region (*traP, trbD, trbG, traV, traR;* Table 3.4) was transformed into the

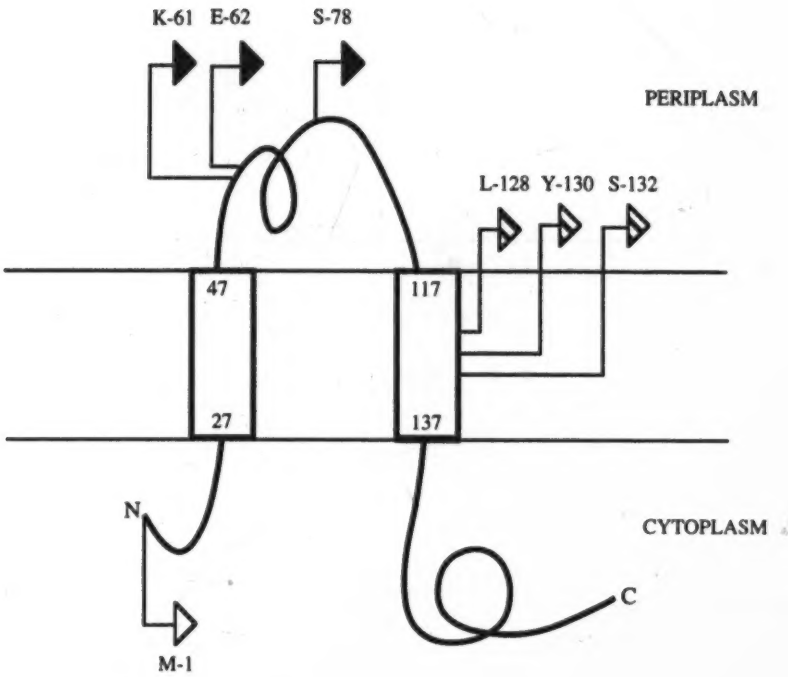
phoA host, *E. coli* CC118 (Table 3.3). Following infection with λTn*phoA*, colonies carrying transpositions on the plasmid were obtained by hyperselection on medium containing kanamycin and the chromogenic substrate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as described in Section 3.4. Dark blue colonies indicating active AP activity, pale blue colonies exhibiting weak activity and white colonies with inactive AP were selected and characterized by restriction mapping and DNA sequencing. Transpositions giving rise to active AP were found to be located in either *traP, traV* which codes for an outer membrane lipoprotein (Doran *et al.*, 1994), or the β-lactamase gene on the vector. Insertions in *traR* resulted in white colonies confirming that TraR is a cytoplasmic protein (Maneewannakul and Ippen-Ihler, 1993). Altogether, seven distinct transpositions in *traP* were identified. Three of these (K_{61}, E_{62}, S_{78}) which exhibited strong enzymatic activity were clustered in the predicted periplasmic loop which separates the two transmembrane segments (Fig. 3.2e). Another three resulting from insertions within the second transmembrane region (L_{128}, Y_{130}, S_{132}) gave rise to weak enzymatic activity. Fusions adjacent to the initiating methionine had no AP activity while insertions to the C-terminus were not obtained. Based on secondary structure predictions and Tn*phoA* analysis, a schematic of the predicted orientation of TraP in the inner membrane together with the sites of Tn*phoA* insertion is presented in Fig. 3.3. In accordance with this prediction, TraP is most likely oriented with its N- and C-termini in the cytoplasm with a loop bracketed by two membrane-spanning segments exposed in the periplasm.

3.2.4 Complementation analysis of pOX38-*traP474* with the homologous alleles from ColB2 and R100-1 plasmids

Since the TraP proteins of F, ColB2 and R100-1 plasmids differ significantly from each other, it was of interest to determine if complementation of pOX38-*traP474* with the homologous genes from ColB2 and R100-1 plasmids would result in phenotypes which differed in their ability to plate f1 phage.

The ability of ColB2 and R100-1 *traP* genes to complement the pOX38-*traP474* mutation was analyzed using chimeric plasmids, pKAR-2 and pKAB-2. These chimeras are derivatives

Fig. 3.3 The predicted orientation of TraP in the inner membrane. The topology of TraP in the inner membrane deduced from computer predictions and Tn*phoA* analysis is shown. Arrows mark the sites of Tn*phoA* insertions. Black arrowheads indicate active alkaline phosphatase (AP) activity, shaded arrowheads represent weak activity and unshaded arrowheads represent no activity. The predicted transmembrane segments are shown as vertical boxes. The letters and numbers refer to the amino acids and their positions in the protein. N and C are the amino and carboxy termini, respectively. Predictions of secondary structure and identification of potential transmembrane segments were done using the GARNIER-ROBSON algorithm (PC/GENE; Garnier *et al.*, 1978) and the method of Rao and Argos (1986).


K-61
E-62
S-78
PERIPLASM
L-128
Y-130
S-132
47
27
117
137
N
CYTOPLASM
C
M-1

of the pUC118 plasmid carrying the *traKBP* region from R100-1 and ColB2, respectively (Table 3.4). An isogenic construct, pKAF-2, carrying the equivalent region in F was also constructed. Matings were performed with *E. coli* XK1200 cells harbouring pOX38-*traP474* and either pKAF-2, pKAB-2 or pKAR-2 as the donors, and *E. coli* MC4100 (Table 3.3) as the recipient. The mating results presented in Table 3.1, show that pKAR-2 and its F counterpart, pKAF-2, restored the mating ability of pOX38-*traP474* from 2.4% to 32% and 35%, respectively. pKAB-2, however, complemented the mutation to 50% of the mating efficiency of pKAF-2 and pKAR-2. This reduction could be due to reduced protein expression in this construct since unlike pKAF-2 and pKAR-2, the orientation of the insert in pKAB-2 is in the opposite direction with respect to the *lac* promoter (Table 3.4).

To determine the effect of ColB2 and R100-1 *traP* alleles on susceptibility to pilus-specific phage infection, f1 and R17 plating efficiency of pOX38-*traP474* in the presence of either one of the chimeric plasmids, was determined by spot test analysis (Section 2.4). The results are shown in Table 3.1. Cells carrying pOX38-*traP474* exhibited 10% of the plating efficiency of the pOX38-Km wild type control. However, when either pKAF-2, pKAB-2 or pKAR-2 was present, wild type levels of phage sensitivity was restored to pOX38-*traP474* cells. These results indicate that TraP does not determine susceptibility to f1 phage infection since despite significant sequence variation, the ColB2 and R100-1 proteins restored complete phage sensitivity to pOX38-*traP474*.

3.2.5 Is TraP directly involved in recipient cell recognition?

Since the *traP474* mutation resulted in decreased mating efficiency despite normal numbers of pili on the cell, it was of interest to determine if TraP might be required for recipient cell recognition. As discussed in Chapter 2, the pyrophosphorylethanolamine (PPEA) moiety on the Heptose I residue in the LPS of recipient cells is required for F plasmid transfer. In order to determine if TraP is involved in LPS receptor recognition, mating experiments with recipient cells carrying mutations in the *rfa* loci (Chapter 2; Fig. 2.1) were carried out.

Table 3.1 Phenotypes of pOX38-*traP474* expressing the homologous genes from ColB2 and R100-1 plasmids

Plasmid[a]	No. of transconjugants[b] (per 100 donors)	% Wild Type (mating efficiency)	Phage sensitivity[c] f1	R17
pOX38-*traP474*	4.36	2.4	++	++
pOX38-Km[d]	90	100	+++	+++
pOX38-*traP474*/pKAF-2	32	35.5	+++	+++
pOX38-*traP474*/pKAR-2	29	32	+++	+++
pOX38-*traP474* /pKAB-2	14.7	16.3	+++	+++

[a]In *E. coli* XK1200.

[b]The recipients for these matings were *E. coli* MC4100.

[c]Phage sensitivity was determined on qualitative spot tests. (+++) 100% wild type; (++) 10% of wild type sensitivity; (+) 1% of wild type sensitivity.

[d]Wild type control

A complete list of the genotypes of recipient cells used in these mating assays are provided in Table 3.3. Since pOX38-*traP474* transfers at detectable levels, its mating efficiency to *rfaP* recipients was measured. If TraP is involved in recognition of the PPEA residue, its mating efficiency to either wild type or *rfaP* recipient would remain approximately the same. In the following mating assay, *E. coli* XK1200 donor cells carrying either pOX38-Km or pOX38-*traP474* were mated with recipients, *E. coli* CS2057 (Δ*rfaGI1*) and CS2058 (Δ*rfa QJ2*) which carry deletions extending from *rfaG* to *rfaI* and *rfaQ* to *rfaJ*, respectively (Chapter 2; Fig. 2.1). These deletions span the *rfaP* gene. The mating efficiencies with respect to the mating efficiency of the wild type control are shown in Table 3.2. As expected, decreased levels of transfer of both pOX38-Km and pOX38-*traP474* are seen with CS2057 and CS2058 as compared to the isogenic wild type control, CS180-2. The pOX38-Km plasmid showed 53% and 70% reduction in mating efficiency with CS2057 and CS2058, respectively as compared to the CS180-2 wild type control. pOX38-*traP474*, however, showed 70% reduction with both CS2057 and CS2058 as compared to CS180-2. In order to determine if supplying the *traP* gene in trans affects the transfer of pOX38-*traP474*, matings were repeated with recipients CS1977 and CS1981. These two strains are λ lysogens of CS2057 carrying the *rfaQJ* and *rfaQGP* genes, respectively, on the prophage. Matings with these strains were carried out at 30°C to prevent the induction of lysis. Since the efficiency of DNA transfer is reduced by 10-fold at 30°C (Frost and Simon, 1993), matings with CS180-2 recipient was also carried out at this temperature to compensate for this reduction. The results are summarized in Table 3.2. The mating efficiency of pOX38-Km with CS1981 (*rfaP*$^+$) was approximately 10-fold higher compared to the mating efficiency obtained with CS1977(*rfaP*$^-$). Similarly, the levels of pOX38-*traP474* transfer also increased when *rfaP* was supplied in trans. These results demonstrate that both plasmids maintain a requirement for *rfaP* activity in the recipient cell, suggesting that TraP is not responsible for the recognition of the PPEA moiety, and thus, is not be involved in recipient cell recognition.

Table 3.2 Transfer efficiency of pOX38-Km and pOX38-*traP474* to recipients carrying mutations in *rfa* loci

Donor Strain *E. coli* XK1200	Recipient *E. coli* (Relevant phenotype)	Transconjugants per 100 donors[a]	% Control[b]
pOX38-Km	CS180-2 (37°C)	100	100
	CS2057 (Δ*rfaGI1*, 37° C)	4.7	4.7
	CS2058 (Δ*rfaQJ2* , 37 °C)	3.0	3.0
pOX38-*traP474*	CS180-2 (37°C)	0.7	100
	CS2057 (Δ*rfaGI1*, 37°C)	0.02	2.9
	CS2058 (Δ*rfaQJ2* , 37°C)	0.02	2.9
pOX38-Km	CS180-2 (30°C)	70	100
	CS1977 (*rfaQ*$^+$*G*$^+$, 30°C)	1.05	1.5
	CS1981 (*rfaQ*$^+$*G*$^+$*P*$^+$, 30°C)	12	17
pOX38-*traP474*	CS180-2 (30°C)	0.440	100
	CS1977 (*rfaQ*$^+$*G*$^+$, 30°C)	0.005	1.1
	CS1981 (*rfaQ*$^+$*G*$^+$*P*$^+$, 30°C)	0.124	28

[a]Mating efficiencies are expressed as the number of transconjugants per 100 donor cells.

[b]Percent mating efficiencies are expressed as the percent relative to the mating efficiency of the CS180-2 wild type control.

3.3 DISCUSSION

In the present study, a comparative analysis of the products of five pilus synthesis genes from F, ColB2 and R100-1 plasmids was undertaken in order to catalogue the extent of sequence variation among these proteins. This work entailed the initial determination of the nucleotide sequences of *traK*, *B* and *P* genes from the R100-1 plasmid and subsequent sequence comparison of TraL, E, K, B and P proteins from the three plasmids by multiple sequence alignment.

Nucleotide sequencing of the *traKBP* region revealed not only that the R100-1 *traK* and *B* genes share considerable homology at the DNA level, but also that the region encoding these genes exhibits a common genetic organization with the F plasmid as predicted by heteroduplex mapping (Sharp *et al.*, 1973). All three genes from both plasmids utilize conserved ribosome binding sites. In addition, the start and stop codons for these genes are closely spaced or overlap each other, an arrangement which is likely to maximize the efficiency of translation via translational coupling (Aksoy *et al.*, 1984).

Multiple sequence alignments revealed minor changes between the TraL, E, K, and B proteins of F and ColB2 plasmids. Specific changes, however, were observed among TraL, E and K proteins in the R100-1 plasmid while its TraB protein contained an additional 11 residues at the C-terminus. The TraP proteins from all three plasmids differed significantly.

Based on secondary structure predictions and Tn*phoA* analysis, the topology of TraP in the inner membrane was determined. TraP is most likely positioned with its N and C-termini in the cytoplasm with two membrane-spanning segments connected by a periplasmic domain. The notion that TraP might be the pilus tip protein was negated by the inability of both R100-1 and ColB2 *traP* alleles to alter the pattern of f1 phage sensitivity of pOX38-*traP474*. In addition, the requirement for a functional *rfaP* product in the recipient cell for the transfer of pOX38-*traP474* also excluded this possibility.

Homologies between the *virB* proteins and the products of *traL*, *E* and *B* suggest that these proteins may form components of the conjugative apparatus. The large number of glycine and proline residues in TraB is characteristic of proteins with an extended conformation such as TonB (Finberg *et al.*, 1995). These proline-rich domains have been postulated to form

stiff 'sticky arms' that mediate interactions with other proteins (Finberg *et al.*, 1995; Williamson, 1994). Recently, the TraB homologue, VirB10, was shown to be tightly associated with the membrane fraction of *A. tumefaciens*, possibly in a complex spanning the cell envelope (Finberg *et al.*, 1995). Since TraB is presumably anchored in the inner membrane with the majority of the protein in the periplasm, it might also mediate interactions among pilus assembly proteins to form a transenvelope connection. Possible translational coupling of *traE, K*, and *B* gene products certainly suggests that these proteins function in a coordinated manner. Evidence for interaction among these proteins, however, still awaits future studies.

3.4 MATERIALS AND METHODS

3.4.1 Bacterial strains, plasmids and general molecular biology techniques

A complete list of genotypes of all the *E. coli* strains and a description of plasmids and transposon used in this study are given in Tables 3.3 and 3.4, respectively. Culture conditions employed are essentially as described in Section 2.4, as are recombinant DNA techniques, procedures for mating assays and determination of transfer efficiencies and PCR.

3.4.2 Cloning and determination of nucleotide sequences of *traK, B* and *P* genes of R100-1

A 3 kb fragment spanning the distal ends of *traE* and *trbD* of R100-1 (corresponding to F coordinates 3437-6424; Frost *et al.*, 1994) flanked by the oligonucleotides KAN-E2 (5'-CGCGGATCCGCGCTGATATCAAACATTACATCCTTA-3') and LFR36 (5'-GAATTCTTCATCACACCGACAG-3'), was amplified by PCR using the R100-1 plasmid as the template. The amplified fragment was cloned into pUC118 (Vieira and Messing, 1987) using the *Bam*HI and *Eco*RI sites provided in the primers to give rise to plasmid, pKAR-2. The R100-1 *traP* gene itself was individually amplified using oligonucleotides KAN-B1 (5'-

Table 3.3 Bacterial Strains used in this Study

Escherichia coli	Relevant genotype	Source/Reference
CC118	Δ*(ara leu)* 7697 Δ*lac* X74 Δ*phoA* 20 *galE galK thi rpsE rpoB argE(am) recA1*	Manoil, 1991
CS180-2	*thr leuB6 proA argE his thi galK l acY trpE mtl xyl ara-14 rpsL* Su^+	C. Schnaitman
CS1977	Δ*rfaGI1*::ΩCm^R of CS180-2 lSG3.4(*rfaQ*$^+$ *G*$^+$)	C. Schnaitman
CS1981	Δ*rfaGI1*::ΩCm^R of CS180-2 lSA4.9 (*rfaQ*$^+$ *G*$^+$ *P*$^+$)	C. Schnaitman
CS2057	Δ*rfaGI1*::Cm^R of CS180-2 *cps-5*::Tn*10*	C. Schnaitman
CS2058	CS2057 but *rfaQJ2*	C. Schnaitman
JC3272	F' *lacΔX74 gal his trp lys rpsL tsx* (λ)	Lab stock
MC4100	F' *araD139 D(argF-lac) U169 rpsL150 relA1 flbB3501 deoC1 ptsF25 rbsR*	C. Manoil (Table 2.4)
XK1200	F' *lacΔU124 Δ (nadA aroG gal attll bio) gyrA*	K. Ippen-Ihler (Table 2.4)
JC3051	F' *lacΔX74his trp rpsL tsx mal* (λ)	Lab stock

Table 3.4 Plasmids and Transposon used in this study

Plasmid	Description of Construct	Source/Comment
pKAF-2	3 kb fragment containing *traK-traP* from F cloned in *Bam*HI/*Eco*RI of pUC118 (Amp^R)	This work
pKAR-2	3 kb fragment containing *traK-traP* from R100-1 cloned in *Bam*HI/*Eco*RI of pUC118 (Amp^R)	This work
pKAB-2	2.9 kb *Eco*RV-*Eco*RI fragment containing *traK-traP* from ColB2 in pUC118 (Amp^R)	This work
pUC118	ColE1 replicon, Amp^R	Vieira and Messing, 1987
pKI250	2.55 kb *Hinc*II fragment of F carrying *traPVR* genes in pBR322 (Tc^R)	Anthony *et al.*, 1996
pBR322	ColE1 replicon, Amp^R, Tc^R	Bolivar *et al.*, 1977
pOX38-Km	Tra^+ $RepFIA^+$ Km^R f1*Hin*dIII fragment of F plus *Hin*dIII fragment of Tn*5*	Chandler and Galas, 1983
pOX38-*traP474*	Tra^+ $RepFIA^+$ Km^R *kan* cassette inserted into *Pst*I of *traP*	This work
R100-1	Tra^+ Cm^R Fa^R Sm^R Sp^R Su^R Tc^R	Lawn *et al.*, 1967 (Table 2.4)
ColB2-Tc	ColB2 Tra^+ Tc^R Cba^R	Finlay *et al.*, 1984 (Table 2.4)
λTn*phoA*	*b*221 *cI*857 *P*am3 with Tn*phoA* in or near *rex*	Manoil, 1991

CGCCCAAGCTTGGGCAGTTTATGTCCGGCAGCC-3') and LFR36. The 799 bp fragment (corresponding to F coordinates 5625-6424; Frost *et al.*, 1994) was subsequently cloned into the *Hin*dIII and *Eco*RI sites in pUC118 and the resulting chimera was named pKAR-P1.

DNA sequencing of the inserts in both recombinant plasmids was carried out using the dideoxy chain termination method of Sanger *et al.* (1977) with a combination of Sequenase Version 2.1 supplied as a kit (US Biochemicals) and a modified dsDNA sequencing protocol described by Kraft *et al.* (1988). Both Universal (5'-GGGTTTTCCCAGTCACGACG-3') and Reverse (5'-AACAGCTATGCCATG-3') primers as well as an internal primer, LFR39 (5'-GGAAGGACAACACCAC-3'), were used to obtain the *traP* sequence. In addition to the universal primer, the following primers nested in the *traK* coding region, KAN-E3 (5'-CTGTTTACCGTTCCCG-3') and PCR4 (5'-CCCTGCTTGTAACCATCAGCC-3') facilitated the completion of its sequence. Four primers, KANB-6 (5'-TTGTACGCACCCGCA-3'), KANB-10 (5'-CCGGAAGGCGAACCA-3'), KANB-3 (5'-GTGGTTCGCCTTCCG-3'), KAN-K1 (5'-GCATGGATGTGTATG-3'), and KAN-E3 were used to obtain most of the *traB* sequence. Determining the remaining sequence in the vicinity of an *Sph*I site in *traB* (corresponding to F coordinate 4446; Frost *et al.*, 1994) necessitated the subcloning of a 2060 bp *Sph*I fragment into pUC118 and subsequent determination of the sequence using the universal primer. The entire nucleotide sequence from *traK* to the distal end of *trbD* is presented in Figure 3.1.

3.4.3 Analyses of DNA and protein sequences

Nucleotide and deduced amino acid sequences were analyzed using the GCG (Devereux *et al.*, 1984) and PC/GENE (IntelliGenetics Inc., 1991) packages. Multiple sequence alignments were generated with the program PILEUP (GCG package) using the Dayhoff 250 PAM matrix (Dayhoff *et al.*, 1978), a gap-weight of 3.0 and a gap-length-weight of 0.2. Predictions of secondary structure for proteins and potential transmembrane segments were made using the GARNIER-ROBSON algorithm (PC/GENE; Garnier *et al.*, 1978) and the method of Rao and Argos (1986).

3.4.4 Construction of pKAF-2 and pKAB-2

The *traK, B,* and *P* genes from both F and ColB2 plasmids were cloned in pUC118 vector in the following manner. A 3 kb fragment encompassing the *traKBP* region (coordinates 3437-6424; Frost *et al.*, 1994) was amplified by PCR using pOX38-Km as the template and the primers, KAN-E2 and LFR36. This fragment was subsequently ligated into the *Bam*HI and *Eco*RI sites in pUC118 to give rise to pKAF-2. The plasmid pKAB-2 was created by subcloning a 2.9 kb *Eco*RV-*Eco*RI fragment containing the *traKBP* region from ColB2 into pUC118.

3.4.5 Generation of *traP* gene fusions by Tn*phoA* transposition

Genetic fusion between the *E. coli* alkaline phosphatase gene, *phoA*, and the *traP* gene was carried out using the λTn*phoA* delivery system described by Manoil (1991). *E. coli* CC118 host cells (Table 3.3) transformed with pKI250 (Table 3.4) were grown at 37°C in liquid LB medium containing 10 mM $MgSO_4$ and 20 µg/ml tetracycline. At early stationary phase, the cells were infected with λTn*phoA* at a multiplicity of infection (MOI) of approximately 1.0. The culture was grown at 30°C for 15 min then it was diluted 1:10 in LB medium to allow outgrowth. To ensure isolation of insertions arising from independent transposition events, diluted aliquots were grown in 10 separate culture tubes at 30°C overnight (Manoil, 1991). Aliquots of 0.2 ml were plated on LB agar containing 300 µg/ml kanamycin and 40 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Boehringer Mannheim). Isolated colonies were replica-plated on LB agar containing 30 µg/ml kanamycin, 20 µg/ml tetracycline and 40 µg/ml BCIP. Plasmid DNA from a large number of blue, pale blue and white colonies were isolated (Sambrook *et al.*, 1989) and digested with *Eco*RI restriction enzyme (Boehringer Mannheim) in order to identify specific transpositions in the *traP* gene. Candidates carrying such transpositions were further sequenced in order to determine the site of Tn*phoA* insertion using a synthetic primer, PhoA (5'-AATATCGCCCTGAGCA-3') which hybridizes to the 5' end of the *phoA* gene in the transposon and directs DNA polymerization through the transposon left ends into the *traP* gene (Manoil, 1991).

3.5 REFERENCES

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CHAPTER 4

Characterization of the nature of the pilus tip: Is there an adhesin?

4.1 INTRODUCTION

Early attempts to study the morphology and composition of F pili entailed both biochemical analysis and direct observation by EM of purified pili (Brinton, 1971; Bradley, 1980; Armstrong *et al.*, 1980). While examination by EM revealed thick flexible filaments with occasional basal knobs, biochemical studies identified only a single type of protein subunit, the pilin, associated with F pili (Section 1.3.3). Unlike adhesive pili which often contain minor proteins at the tip, the nature of the tip of most conjugative pili remains obscure. However, with the exception of pili of the IncF complex, most conjugative pili possess pointed or tapered tips suggesting a conical arrangement of either pilin subunits or another minor protein at the tip (Frost, 1993). The apparently 'blunt' nature of the F pilus tip, is probably due to a helical arrangement of subunits too shallow to be visible. Although no evidence for a unique minor protein at the tip exists, the attachment of M13 phages to pili of both F and pED208 plasmids whose pilin sequences share little homology (Section 1.4.5; Datta, 1975), argue for this possibility. In addition, the findings in Chapter 2 where an F *traA* null mutant expressing either ColB2 or R100-1 pili still retained an F-like phenotype also agrees with this notion. Thus, the objective of the work described here was to explore the possibility that the F pilus tip contains a unique protein that acts as the receptor for M13 phage.

The strategy used to physically isolate the putative pilus tip protein entailed the use of a fusion protein comprising the pilus attachment domain of the M13 minor coat protein, pIII (Section 1.6.2). The chimeric plasmid, pIII-STE, encoding the fusion protein was obtained from F. Breitling and S. Dubel (University of Heidelberg, Germany). A physical map of the plasmid and all the relevant features of the fusion protein are shown in Fig. 4.1A and B, respectively. The fusion protein, pIII-STE, contains the N-terminal 390 aa of pIII (van Wezenbeek *et al.*, 1980) fused in-frame to the coding region of core-streptavidin (Pahler *et al.*, 1987). This region of pIII contains all the features necessary for binding the pilus tip and TolA (Section 1.6.2). In addition, since the C-terminal hydrophobic residues of pIII were deleted during the construction of the fusion, the recombinant protein can be purified in a highly soluble form. Core-streptavidin is a 125-127 aa cleavage product arising from both N-terminal and C-terminal processing of full length (159 aa) streptavidin (Pahler *et al.*,

Fig. 4.1 A physical map of pIII-STE chimeric plasmid (A) and functional features of pIII-STE fusion protein (B). P/O upstream of the fusion protein coding sequences is the *lac* promoter and operator sequences for IPTG-inducible expression of the fusion. *pelB* leader is the *Erwinia carotovora* pectate lyase leader peptide engineered to allow secretion of the fusion into the periplasmic space. N-pIII is the 390 aa N-terminal domain of pIII responsible for pilus binding. The *Bam*HI spacer links N-pIII to the coding regions of core-streptavidin. The 5-His at the C-terminus is the 5-histidine tag for the purification of pIII-STE on a Ni^{2+} affinity column. Ap^R and ori denote β-lactamase gene and ColE1 origin of replication, respectively.

Fig. 4.1A

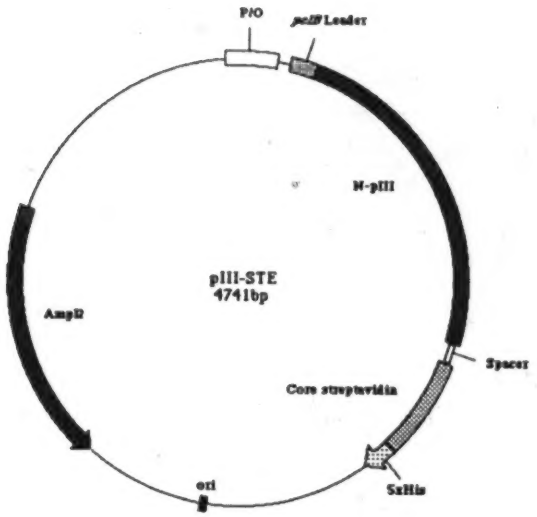
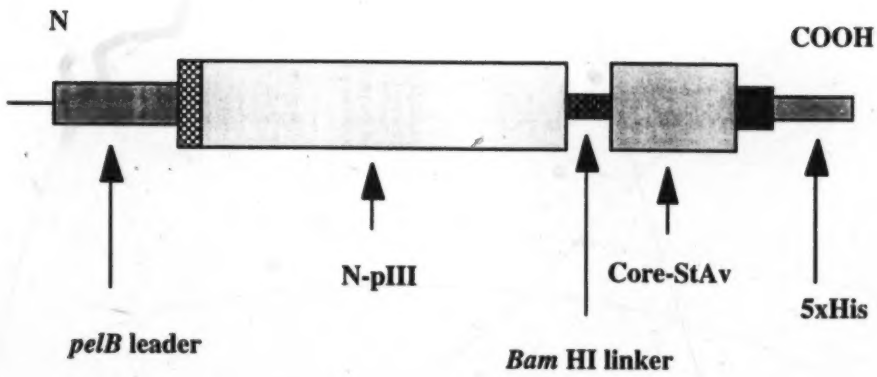


Fig. 4.1B



1987). Since streptavidin naturally exists as a tetramer, the number and spatial arrangement of the pIII moiety in the tetrameric fusion is expected to mimic its actual configuration on the phage particle (Section 1.6.2). The gene fusion was constructed in the expression vector, pOPE51 (Kipriyanov *et al.*, 1994), in which recombinant proteins are expressed from the IPTG-inducible *lac* promoter. In addition to the *pelB* leader sequence, which allows secretion of the mature protein into the periplasm (Lei *et al.*, 1987), the vector also incorporates a 5-histidine tag at the C-terminus to facilitate purification of the recombinant protein by Ni^{2+} affinity chromatography (Dubel *et al.*, 1992).

Previous attempts to purify large quantities of F pili free from cellular debris have proven difficult due to their smaller numbers (1-2 pili/cell) and their tendency to adhere to cells (Paranchych, 1982). To circumvent this problem, pili were purified from cells harbouring the pOX38-*tra715* plasmid in which the expression of the entire *tra* operon is placed under the control of the bacteriophage T7 promoter sequences (Maneewannakul *et al.*, 1992). In this construct, pili can be labelled with radioactivity not only as a means to increase the sensitivity of detection and thus compensate for the deficiency in yield, but also to avoid interference from contaminating unlabelled cellular proteins.

Using [^{35}S]-methionine labelled F pili and pIII-STE fusion as the high affinity ligand for the putative pilus tip protein, an affinity purification procedure was developed in order to isolate the M13 phage receptor at the pilus tip. The findings of this work are presented in this chapter.

4.2 RESULTS

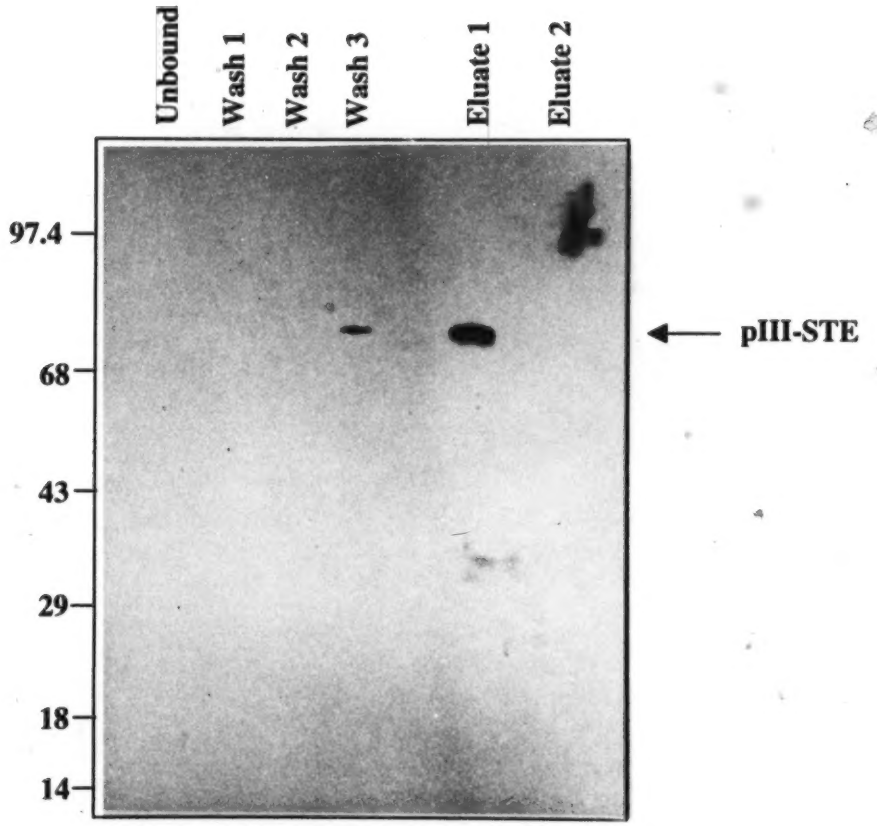
4.2.1 Affinity Purification of pIII-STE fusion protein

In order to achieve high levels of expression of the fusion protein, *E. coli* K802 (Woods, 1968) cells were transformed with plasmids pIII-STE and pDMI.1. The latter is a compatible plasmid which overproduces the *lac* repressor (Lanzer, 1988) and thus represses the expression of the fusion protein until induction. Liquid cultures of K802 cells carrying the two plasmids were induced with 100 µM IPTG for 3 hours. The periplasmic fraction was prepared by subjecting the cells to osmotic shock and the fusion protein in the osmotic shock fluid was affinity purified using Ni^{2+}-NTA (nickel-nitrilotriacetate) resin as described in Section 4.4. The adsorbed pIII-STE fusion protein was eluted with an imidazole gradient and the products from the affinity purification were analyzed by SDS-PAGE (Section 4.4). The presence of pIII-STE protein was confirmed by Western blotting (Section 4.4) using a polyclonal rabbit antiserum raised against streptavidin. Figure 4.2 shows a Western blot of fractions derived from different steps in the purification process. The prominent band and the minor band at approximately 68 kDa in the first eluate and the third wash fractions, respectively, correspond to the monomeric form of pIII-STE protein. Although the calculated mass of pIII-STE is only 54 kDa (Breitling and Dubel, unpublished data), the apparent increase in its molecular weight is due to the anomalous migration of the pIII moiety on SDS-polyacrylamide gels (Goldsmith and Konigsberg, 1977). An estimate of the amount of purified protein, as determined by comparison with known amounts of streptavidin on a Western blot, revealed a yield of 5 µg/litre of cell culture. The yield, albeit low, contains stable and fully functional fusion protein with no signs of immediate degradation as shown below.

4.2.2 Functional characterization of pIII-STE fusion protein

The ability of purified pIII-STE fusion to attach to the F pilus tip was initially determined indirectly by testing for its ability to inhibit the infection of piliated F^+ cells by the helper phage, M13KO7 (Vieira and Messing, 1987). Since this M13 derivative transduces a

Fig. 4.2 Affinity purification of pIII-STE fusion. *E. coli* K802 cells carrying the plasmids pIII-STE and pDMI.1 were induced with 100 μM IPTG for production of the fusion protein. The periplasmic fraction was prepared by subjecting the cells to osmotic shock and the pIII-STE protein in the osmotic shock fluid was affinity purified on Ni^{2+}-NTA resin as described in Section 4.4. After removal of the unbound material by centrifugation, the resin was washed successively with Buffers A and B containing 10 mM and 30 mM imidazole, respectively to remove non-specifically bound material. The adsorbed fusion protein was eluted from the resin by washing with Buffer C containing 400 mM imidazole followed by Buffer D containing 50 mM EDTA. The unbound, wash and eluate fractions were separated on a 10% SDS-polyacrylamide gel. The pIII-STE fusion was detected on a Western blot with polyclonal streptavidin-specific antiserum and anti-rabbit-HRP conjugate secondary antibodies and chemiluminescent reagents. Wash 1 and 2 correspond to the first two washes with Buffer A while wash 3 is with Buffer B. Eluate 1 and 2 are washes done with Buffers C and D, respectively. The positions of molecular mass markers in kDa are indicated on the left. The position of pIII-STE is marked with an arrow.


Unbound
Wash 1
Wash 2
Wash 3
Eluate 1
Eluate 2
97.4
68
43
29
18
14
pIII-STE

kanamycin resistance marker upon entry into host cells, the efficiency of its infection can be easily determined by scoring the number of kanamycin resistant colonies. *E. coli* DH5α (Km^S, F pili$^-$) and DH5αF' (Km^S, F pili$^+$) cells were infected with M13KO7 following preincubation with 5 pmol of pIII-STE fusion or an equal amount of bovine serum albumin (BSA) as a negative control. The efficiency of M13KO7 infection recorded as percent infection is tabulated in Table 4.1. Kanamycin-resistant colonies were obtained only when DH5αF' host cells were used indicating that M13KO7 infection is F pilus-specific. The percent infection values for cells preincubated with pIII-STE fusion protein are approximately 100-fold lower than the values for cells preincubated with BSA. These results indirectly demonstrate that pIII-STE can effectively compete with M13KO7 phage for F pilus tip attachment.

In order to demonstrate that pIII-STE specifically binds to the F pilus tip, piliated DH5αF' cells were preincubated with 5 pmol of the fusion protein and the cells were prepared for immunogold electron microscopy as described in Section 4.4. The fusion protein was detected with polyclonal streptavidin-specific antiserum and anti-rabbit secondary antibodies conjugated to colloidal gold. Fig. 4.2 shows a composite of four electron micrographs depicting F pili with one (B), two (C), three (A) and four (D) gold particles at the tip. Of a total of 47 pili examined, approximately 14 pili (30%) had between 1-4 gold particles positioned at their tips. Immunogold electron microscopy in the absence of pIII-STE fusion did not reveal deposition of gold particles at the pilus tip (data not shown). These results directly demonstrate that the fusion protein binds specifically to the F pilus tip. Previous reports have documented that a maximum of three M13 phage particles can attach simultaneously to the pilus tip (Marvin and Hohn, 1969). The presence of 1-4 gold particles at the tip suggests that the fusion binds in a manner similar to the phage itself and that the 4 gold particles could have arisen from tetramer formation by pIII-STE fusion protein.

Table 4.1 Inhibition of M13KO7 infection by pIII-STE fusion

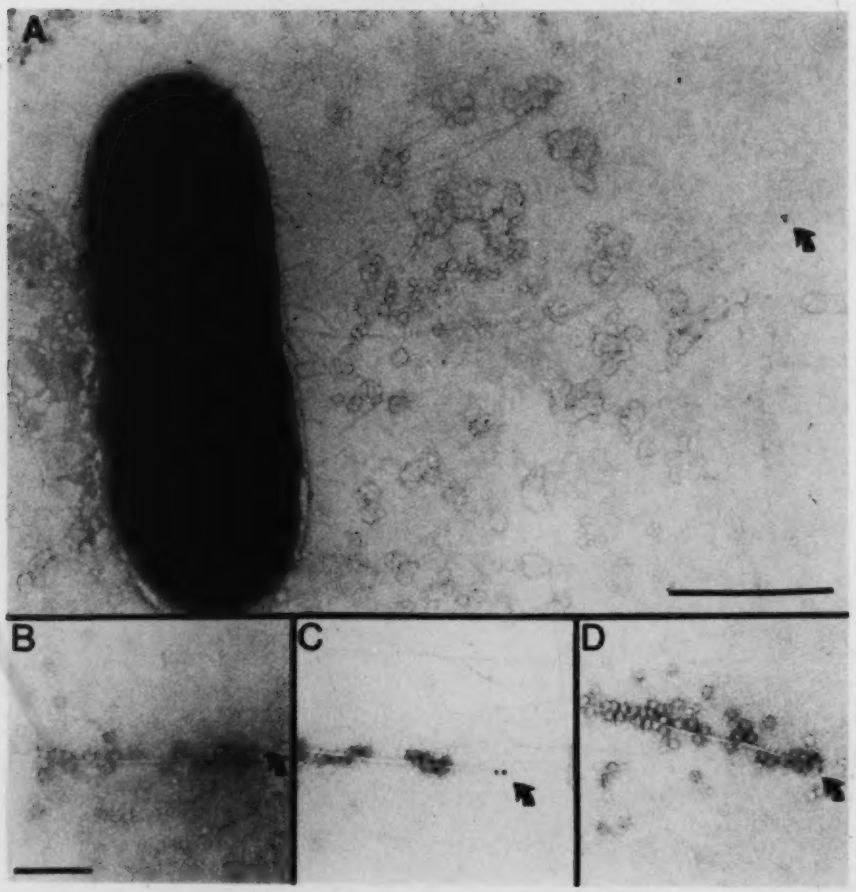
Strain	Total count[a] # colonies		Km^R colonies[b] # colonies		% Infection[c]	
(-) pIII-STE						
DH5α	$5.1x10^7$	$3.2 x10^7$	0.0	0.0	0.0	0.0
DH5αF'	$1.1x10^7$	$3.4x10^7$	$5.8x10^5$	$1.0x10^6$	5.8	3.7
(+) pIII-STE						
DH5α	$6.0x10^7$	$3.2x10^7$	0.0	0.0	0.0	0.0
DH5αF'	$2.2x10^7$	$1.6x10^7$	$7.8x10^3$	$6.3x10^3$	0.04	0.06

[a] Total number of cells was determined by plating various dilutions of uninfected cells on LB agar.

[c] The % infection was calculated as the ratio of kanamycin^R colonies obtained to the total number of cells (b/a).

Data were obtained from two independent experiments.

Fig. 4.3 Binding of pIII-STE fusion to the F pilus tip. *E. coli* DH5αF' cells were preincubated with 50 pmol of pIII-STE fusion and the cells were examined by immunogold electron microscopy as described in Section 4.4 to determine the ability of the fusion protein to bind to the pilus tip. The pIII-STE fusion was identified with polyclonal streptavidin-specific rabbit antiserum and secondary anti-rabbit antibodies conjugated to colloidal gold. Arrows mark the positions of 1, 2, 3 and 4 gold particles (B, C, A and D, respectively) at the pilus tip. The pili depicted in B, C and D are laterally coated with R17 phage. The size bar represents 1 μm in A and 0.5 μm in B, C and D. Total magnifications are 23 630x (A) and 59 260x (B,C, and D).

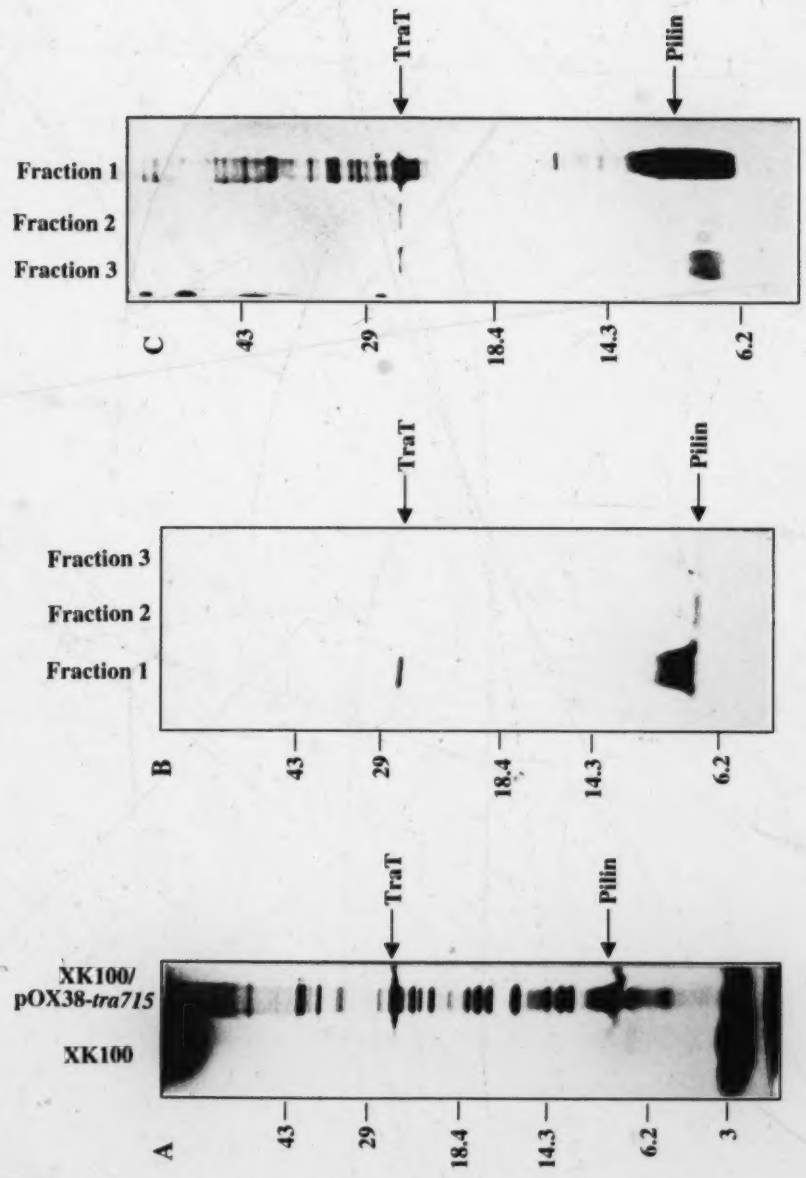

A
B
C
D

4.2.3 Purification of ^{35}[S]-methionine labelled F pili

E. coli XK100 cells (Section 4.4) harbouring the pOX38-*tra715* plasmid described in Section 4.1, were used for the purification of radioactively labelled F pili. Despite the high processivity and efficiency of the T7 RNA polymerase/promoter system which controls the expression of the *tra* operon in pOX38-*tra715*, cells harbouring this plasmid have been shown to synthesize normal amounts of transfer proteins without any notable deleterious effects to the host. In addition, this plasmid mediates efficient DNA transfer and produces on average one pilus per cell which attaches both f1 and R17 phages well (Maneewannakul, *et al.*, 1992). *E. coli* XK100 was used as the host since it carries a T7 RNA polymerase gene under the control of the *lacUV5* promoter on a DE3 lysogen and thus allows the selective radioactive labelling of transfer proteins upon induction with IPTG and addition of rifampicin to arrest host cellular transcription (Maneewannakul, *et al.*, 1991). Although XK100 cells produce both fimbriae and flagella, they were used in this study since attempts to supply the T7 RNA polymerase in trans on plasmids such as pKI501 (Maneewannakul *et al.*, 1992) and pGP1-2 (Tabor and Richardson, 1985) in *E. coli* bald strains (*fimbriae*$^-$ *flagella*$^-$), resulted in very poor labelling. ^{35}[S]-methionine was chosen for labelling the *tra* proteins since all fifteen of the transfer proteins required for pilus synthesis contain at least one methionine residue in their mature products (Frost *et al.*, 1994).

A liquid culture of XK100 cells carrying pOX38-*tra715* was grown to late logarithmic phase. The cells were harvested and resuspended in minimal medium and induced with IPTG for expression of T7 RNA polymerase as described in Section 4.4. Following the addition of rifampicin, the *tra* proteins were radioactively labelled with ^{35}[S]-methionine. F pili from the culture supernatant were collected by precipitation with 2% polyethylene glycol 8000 (PEG) and 0.5 M NaCl then separated on a CsCl density gradient as described in Section 4.4. Individual fractions from the gradient were collected and the amount of radioactivity in each fraction was determined. Fractions containing significant amount of radioactivity were separated on an SDS-polyacrylamide gel and pilus proteins were visualized by autoradiography. Figure 4.4A is an autoradiograph of an SDS-polyacrylamide

Fig. 4.4 Purification of radioactively labelled F pili. **A**. Autoradiography of transfer proteins in pOX38-*tra715*. Total cellular proteins from XK100 cells and XK100 cells harbouring pOX38-*tra715* were labelled with [^{35}S]-methionine after the addition of IPTG and rifampicin. Approximately $1x10^7$ cells were solublized in SDS sample buffer and total cellular proteins were separated on a 15% SDS-polyacrylamide gel. **B**. Autoradiography of [^{35}S]-methionine labelled pili purified from pOX38-*tra715*/XK100 cells. Pili from the culture supernatant were precipitated with 2% PEG 8000 and 0.5 M NaCl and banded on a CsCl density gradient. 5 µl aliquots of the three fractions containing significant amounts of radioactivity were separated on a 15% SDS-polyacrylamide gel. **C**. SDS-PAGE of 20 µl aliquots of the three fractions as in **B** visualized by silver staining. Arrows mark the positions of TraT and pilin. Molecular mass markers (kDa) are shown on the left.


TraT
Pilin
Fraction 1
Fraction 2
Fraction 3
C
43
29
18.4
14.3
6.2
TraT
Pilin
Fraction 3
Fraction 2
Fraction 1
B
43
29
18.4
14.3
6.2
TraT
Pilin
XK100/
pOX38-tra715
XK100
A
43
29
18.4
14.3
6.2
3

gel of total cellular proteins.isolated from either XK100 cells or XK100 cells harbouring pOX38-*tra715* in which the proteins were labelled with ^{35}[S]-methionine. Radioactively labelled proteins are visible only in XK100 cells carrying pOX38-*tra715* plasmid but not in XK100 cells, indicating that the radioactive labelling is specific for plasmid-encoded proteins. Figure 4.4B is an autoradiograph of an SDS-polyacrylamide gel containing three fractions collected from the CsCl density gradient which contain pili. Pilin is visible as a discrete band at 7.2 kDa in all three fractions with Fraction 1 containing the most significant amount. The band at 26 kDa (Fraction 1) was later confirmed immunologically to be the outer membrane protein, TraT (data not shown). Figure 4.4C shows an SDS-polyacrylamide gel of the purified pili corresponding to the three fractions as in 4.4B visualized by silver staining. Apart from the two prominent bands corresponding to pilin and TraT, other minor bands possibly contaminating outer membrane proteins, are also visible. Examination by electron microscopy of pili in Fraction 1 revealed the presence of rope-like structures characteristic of F pili as shown in Fig. 4.4D. The ability of purified pili to attach M13KO7 phage and thus block the infection of DH5αF' cells was also tested as described previously for pIII-STE (Section 4.2.3). Five µl of purified pili from Fraction 1 was mixed with approximately $6x10^7$ pfu of M13KO7. This mixture was added to DH5αF' cells and the percent infection was determined by counting the number of Kanamycin^R colonies as described earlier. The results presented in Table 4.2 show that preincubation of M13KO7 with purified F pili reduces the percent infection by approximately 78-fold demonstrating the ability of purified pili to attach the phage.

Fig. 4.4D Purified [^{35}S]-methionine labelled F pili. F pili from Fraction 1 were negatively stained with phosphotungstic acid and were examined by TEM as described in Section 4.4. Arrows show rope-like structures corresponding to aggregates of F pili. The size bar represents 1 µm and the total magnification is 17 000x.

D

Table 4.2 Inhibition of M13KO7 infection by purified F pili

Strain	Total count # colonies		Km^R colonies # colonies		% Infection[a]	
DH5αF'	$2.5x10^7$	$4.2x10^7$	$8.2x10^5$	$4.3x10^5$	3.28	1.02
DH5αF' + pili	$6.8x10^7$	$5.0x10^7$	$2.4x10^4$	$8.2x10^3$	0.035	0.016
DH5αF' + sonicated pili	$2.1x10^7$	$5.5x10^7$	$6.1x10^3$	$6.4x10^2$	0.029	0.011

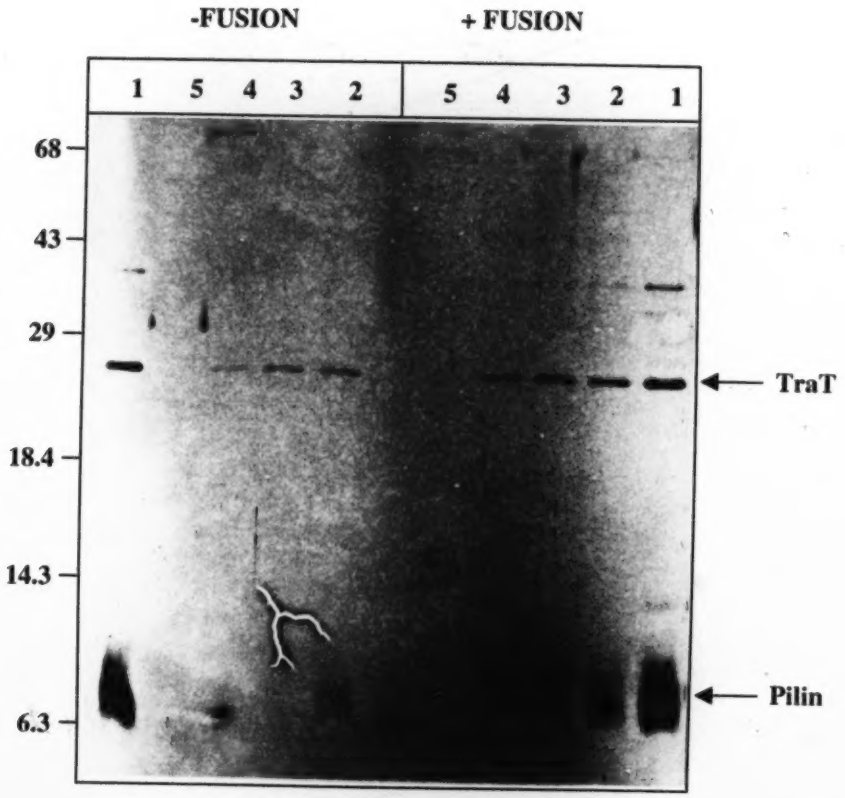
[a] % infection was determined as for Table 4.1

4.2.4 Preliminary analysis to determine interactions between pIII-STE and putative pilus tip protein by affinity purification

In order to isolate the putative pilus tip protein, affinity purification was performed between pIII-STE fusion bound to Ni^{2+}-NTA resin by the His tag at its C-terminus and sheared ^{35}[S]-methionine labelled pili. Radioactively labelled pili were sheared by sonication to isolate short fragments with intact tip structures and to reduce the amount of excess pilus filaments that are not involved in pIII-STE binding. Pili sheared in this manner still retained the ability to attach M13KO7 as efficiently as intact pili as shown by their ability to the reduce phage infection (Table 4.2). Approximately 100 ng of pIII-STE fusion protein was initially immobilized on Ni^{2+}-NTA resin. The resin was mixed with approximately 750 ng of sheared pili to allow interactions between the pilus tip and adsorbed pIII-STE to occur. A negative control in which sheared pili mixed with Ni^{2+}-NTA resin in the absence of the fusion protein was carried through the same procedures to determine the extent of non-specific binding of pili to the resin.

The unbound material was subsequently removed by centrifugation and the resin was successively washed with buffers containing 10 mM and 30 mM imidazole, respectively in order to remove non-specifically bound material. Finally, the adsorbed fusion protein was eluted from the resin by successive washing with buffers containing 400 mM imidazole and EDTA. The unbound, wash and eluate fractions were analyzed on two 15% SDS-polyacrylamide gels. One gel was visualized by silver staining while the other was prepared for 'Far Western' analysis as described in the following section. Figure 4.5 shows the silver-stained SDS-polyacrylamide gel. The products obtained from the affinity purification in the presence of pIII-STE (+ Fusion) and the negative control (- Fusion) are shown. The majority of pilin is seen in the unbound fraction (lane 1) while lesser amounts are present in the first two washes (lanes 2 and 3) as well as in the first eluate fraction (lane 4) in affinity purification with pIII-STE. Corresponding amounts of TraT are also observed in all four fractions. Apart from pilin and TraT, a band at approximately 35 kDa is also visible. This product corresponds to the partially denatured pilin pentamers as previously confirmed by Western blotting with TraA-specific antiserum (data not shown). Although equal amounts of pili were

Fig. 4.5 Analysis of pIII-STE-pilus tip interactions by affinity purification. Specific interactions between pIII-STE and pilus tip was investigated by affinity purification of pili with pIII-STE preadsorbed on Ni^{2+}-NTA resin. As described in Section 4.4, after removing the unbound material the resin was washed with buffers containing two concentrations of imidazole prior to successive elution of the adsorbed pIII-STE with buffers containing 400 mM imidazole and EDTA. The products from the affinity purification were separated on a 15% SDS-polyacrylamide gel and were visualized by silver staining. (+ Fusion) and (- Fusion) refer to affinity purification in the presence and absence of pIII-STE fusion protein, respectively. Lanes 1-5 represent the unbound, wash 1 (10 mM imidazole), wash 2 (30 mM imidazole), eluate 1 (400 mM imidazole) and eluate 2 (50 mM EDTA), respectively. Arrows indicate the positions of TraT and pilin. The molecular mass markers in kDa are shown on the left.


-FUSION
+ FUSION
1 5 4 3 2
5 4 3 2 1
68
43
29
18.4
14.3
6.3
TraT
Pilin

used in the negative control, considerably lower amounts of pilin are seen in both the wash and eluate fractions (lanes 2, 3 and 4). This was later determined to be due to increased non-specific binding of pili to the Ni^{2+}-NTA resin in the absence of adsorbed pIII-STE fusion protein. Based on Fig. 4.5 any minor proteins corresponding to the pilus tip adhesin which have affinity for pIII-STE fusion are either absent or could not be detected.

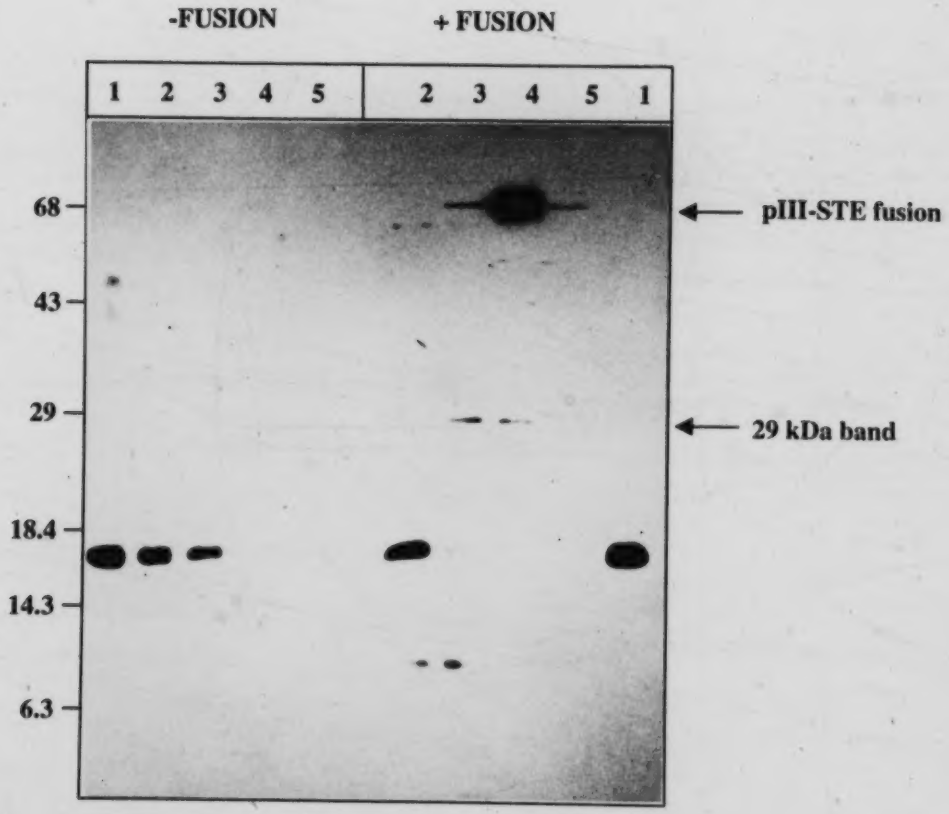
4.2.5 Analysis of 'pilus associated proteins' with affinity to pIII-STE by 'Far Western' technique

Since specific interactions between pIII-STE and 'pilus associated proteins' could not be detected by direct visualization of the products from the affinity purification, the 'Far Western' technique (Phizicky and Fields, 1995) was performed in order to increase the sensitivity of detection. Unlike the affinity purification in which the interaction between pIII-STE fusion and the pilus tip occurs under native conditions, the 'Far Western' technique identifies interactions between pIII-STE and its denatured ligand immobilized on a membrane. Thus, this technique would fail to identify the pIII-STE receptor if the receptor is in a unique conformation at the pilus tip or if the binding site is formed through the cooperative association of more than one protein.

Following SDS-PAGE under reducing conditions, the products from the affinity purification described in the previous section, were transferred to a PVDF membrane. After blocking of free binding sites and partial renaturation of proteins, the membrane was incubated with pIII-STE fusion protein. This incubation allows pIII-STE to bind to its potential partners which are immobilized on the membrane. The membrane was subsequently incubated with anti-streptavidin primary antibody and HRP-conjugated secondary anti-rabbit IgG. The membrane was treated with chemiluminescent substrates to detect signals at positions where pIII-STE is bound to another protein (Section 4.4).

Figure 4.6A shows the results from the 'Far Western' analysis. The pIII-STE fusion itself is visible as a distinct band at 68 kDa predominantly in the first eluate (lane 4) and as minor bands in the second wash and eluate fractions (lanes 3 and 5, respectively) of the + Fusion section. A second prominent band at 17 kDa indicates that the pIII-STE fusion

Fig. 4.6A 'Far Western' analysis of pIII-STE-pilus tip interactions. Affinity purification to identify the putative pilus tip protein was carried out using pIII-STE fusion immobilized on Ni^{2+}-NTA resin and radioactively labelled F pili as described in Section 4.4. The conditions for washing and elution are as described for Fig. 4.2. The unbound, wash and eluate fractions were separated on a 15% SDS-polyacrylamide gel. Following transfer of the proteins to a PVDF membrane, the membrane was incubated with pIII-STE fusion prior to incubation with polyclonal anti-streptavidin antiserum and anti-rabbit IgG-HRP conjugate. The signals were detected by incubating the membrane with chemiluminescent substrates (Section 4.4). + Fusion and - Fusion refer to affinity purification in the presence and absence of pIII-STE, respectively. Lanes 1-5 represent the unbound, wash 1 (10 mM imidazole), wash 2 (30 mM imidazole), eluate 1 (400 mM imidazole) and eluate 2 (50 mM EDTA), respectively. The positions of pIII-STE and the 29 kDa band are marked with arrows. The molecular mass markers in kDa are shown on the left.


-FUSION
+ FUSION
1 2 3 4 5
2 3 4 5 1
68
43
29
18.4
14.3
6.3
pIII-STE fusion
29 kDa band

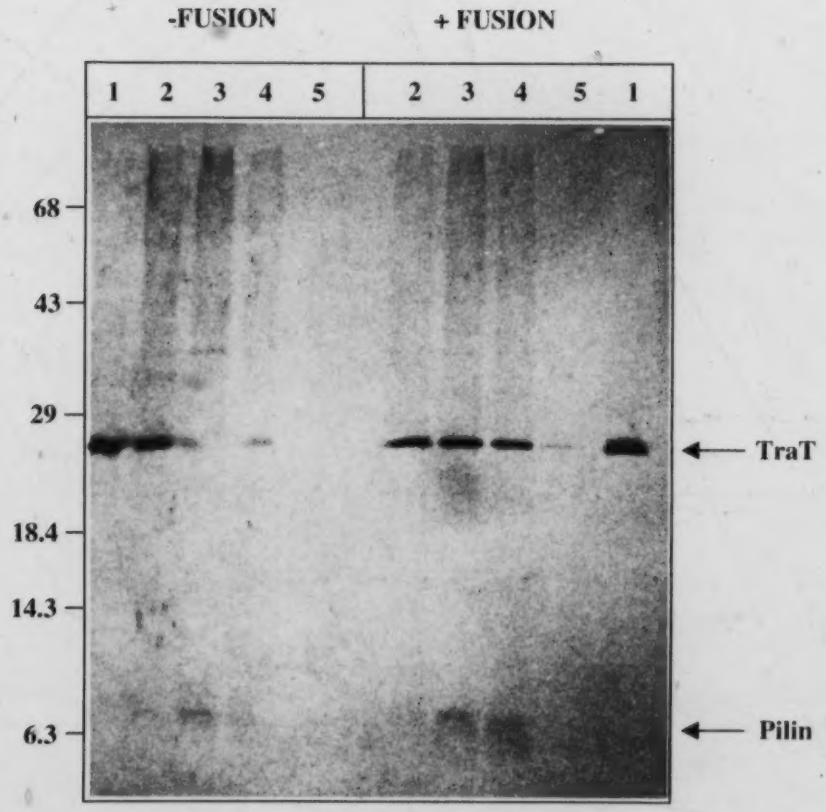
A

exhibits affinity for a protein of unknown nature (+ Fusion, lanes 1 and 2; - Fusion, lanes 1, 2 and 3). Since this band is present only in the unbound and wash fractions but not in the eluate fractions, it was considered a false positive. Furthermore, the appearance of a signal at this position could not be confirmed in subsequent attempts. A unique band at 29 kDa is visible in the second wash and the first eluate fractions (+ Fusion; lanes 3 and 4). A similar band, though not visible in Fig. 4.6A, was also present in the first wash fraction of the negative control (- Fusion, lane 2) in the original Western blot. The appearance of this band corresponds to conditions under which the fusion protein itself is eluted from the Ni^{2+}-NTA resin (30 mM and 400 mM imidazole), suggesting that the 29 kDa protein may be bound to the resin via its interaction with the pIII-STE fusion protein. No signal corresponding to either pilin or TraT was detected in Fig. 4.6A indicating that pIII-STE does not interact with either TraT or pilin monomers. As a control, a second membrane with identical samples was incubated with streptavidin; with the exception of a signal corresponding to pIII-STE, no other signals was detected (data not shown).

4.2.6 Analysis of products of pIII-STE-pilus interactions by autoradiography

Because of the high degree of sensitivity associated with detection of radioactively labelled products, the membrane from the 'Far Western' analysis was dried and the proteins were visualized by autoradiography. This was done firstly to determine if a radioactively labelled product that interacts with pIII-STE is present in purified pili and secondly, to determine if the 29 kDa product that has affinity for pIII-STE in the 'Far Western' analysis is a radioactively labelled *tra* protein. Figure 4.6B shows the autoradiogram of the dried membrane. Two distinct bands corresponding to TraT and pilin are the only radioactively labelled products visible. However, the amount of pilin present is significantly less in comparison to the amount seen in Fig. 4.5. This reduction could be due to the preferential loss of low molecular weight products during repeated washings in the 'Far Western' analysis. To circumvent this problem an SDS-polyacrylamide gel containing identical samples as in Fig. 4.5, was dried and the proteins were visualized directly by autoradiography without prior treatment by 'Far Western' analysis. Identical results as in Fig. 4.5 were obtained (data not

Fig. 4.6B Analysis of pIII-STE-pilus tip interactions by autoradiography. The membrane from the 'Far Western' analysis was dried and autoradiographed to identify the radioactively labelled products. Lane designations are as described for Fig. 4.6A. Arrows indicate the positions of TraT and pilin. The molecular mass markers in kDa are shown on the left.



B

shown). A 29 kDa product with affinity for pIII-STE in the 'Far Western' analysis (Fig. 4.6A) could not be identified either in Fig. 4.5 or in Fig. 4.6B. Neither prolonged exposure nor examination of the membrane in Fig. 4.6B by phosphorimager analysis revealed a radioactively labelled 29 kDa product. Since none of the fifteen *tra* genes involved in pilus synthesis encode a product with a predicted molecular weight of 27 000-30 000, the 29 kDa band most likely is not a *tra* protein. The absence of a signal at the corresponding position in radioactively labelled pili also supports this notion. The 'Far Western' analysis was repeated twice to ensure reproducibility and in both instances pIII-STE reacted with a 29 kDa product. These results indicate that pIII-STE exhibits affinity for a 29 kDa host-encoded product of unknown nature which could have been derived from outer membrane contamination in purified pili.

4.2.7 Analysis of pIII-STE-pilus interactions by affinity purification using biotinylated acrylic beads

When the affinity purification to determine interactions between pIII-STE and pilus tip was performed using the Ni^{2+}-NTA resin, a significant amount of sheared pili was found to be non-specifically adsorbed to the resin. Since non-specific interactions between sheared pili and the resin could have contributed to the loss in the amount of sheared pili recovered, biotinylated acrylic beads and biotinylated agarose matrix were used to circumvent the problem. However, only the former significantly reduced the non-specific binding of pili; as such, only the results obtained from the affinity purification using acrylic beads are presented below. The pIII-STE fusion was preadsorbed to biotinylated acrylic beads via the streptavidin moiety at its C-terminus (Fig. 4.1B) as described in Section 4.4. Sheared pili were mixed with the beads and interaction between pIII-STE and pilus tip was allowed to proceed as described earlier. Following binding, the beads were washed successively with PBS containing 0.5 M NaCl to remove non-specifically bound material. In order to elute the pilus material that is bound to the adsorbed pIII-STE fusion, the beads were subsequently heated to 100°C in SDS sample buffer (Section 4.4) prior to SDS-PAGE analysis. Since extremely stringent conditions are required to disrupt streptavidin-biotin interactions, this treatment does not

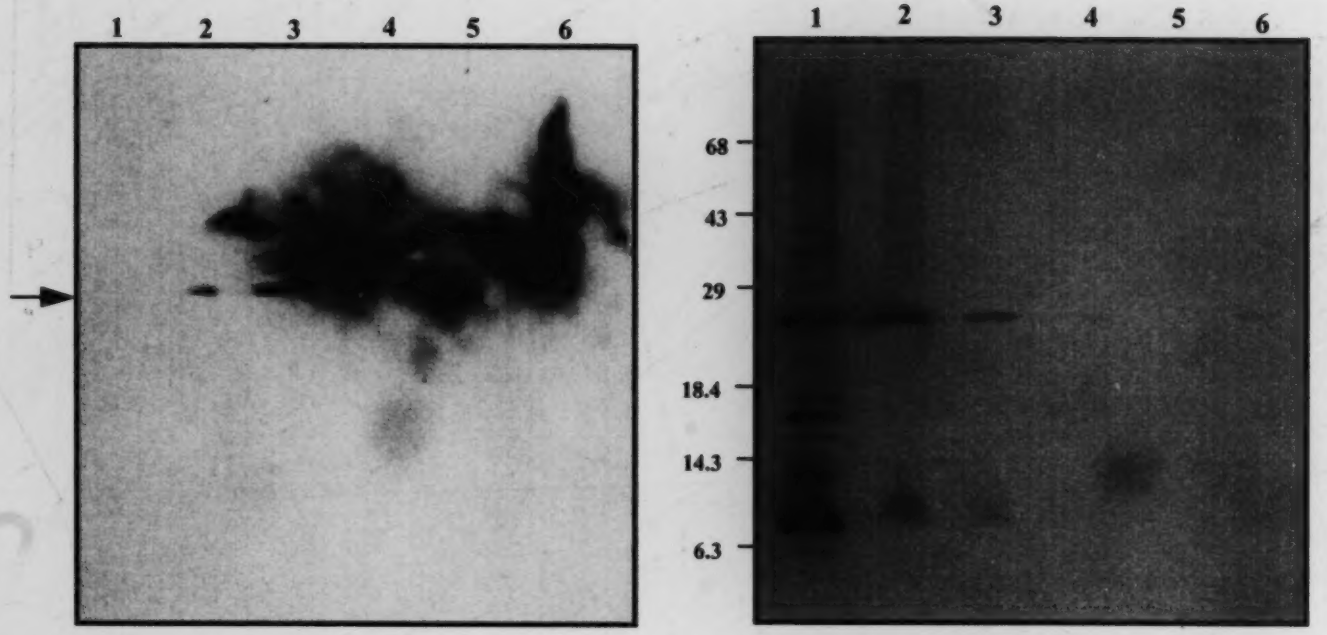
remove pIII-STE fusion from the beads. The products were analyzed by 'Far Western' as described previously. Figures 4.7A and B are the immunoblot from the 'Far Western' analysis and the autoradiogram of the membrane, respectively. Consistent with the results obtained previously, pIII-STE shows affinity for a 29 kDa product (Fig. 4.7A, lanes 2, 3, 4 and 6). This product is visible predominantly in the wash fractions probably because the washing conditions were too stringent. Lack of a signal with total cellular proteins from XK100 cells harbouring pOX38-*tra715* (Fig. 4.7A and B, lane 1) indicates that pIII-STE fusion does not interact with any cellular or *tra* proteins. As suspected, examination of the autoradiogram in Fig. 4.7B did not reveal a radioactively labelled 29 kDa band (lanes 2-6). Most of TraT and pilin are seen in the unbound and the first wash fractions (Fig. 4.7B, lanes 2 and 3) and residual amounts are also present in the eluate (Fig. 4.7B, lane 6). Other minor proteins associated with the pilus could not be identified. Collectively, these results suggest that a unique pilus tip protein which mediates interactions with pIII-STE most probably does not exist.

4.3 DISCUSSION

Based on genetic complementation studies and M13 phage sensitivity profiles conferred by pili with non-homologous pilin sequences, the possibility of a unique pilus tip protein had been suggested (Anthony *et al.*, 1994). To explore this notion further, the pIII-STE fusion protein comprising the N-terminus of pIII and core-streptavidin, was used as a high-affinity ligand to isolate the M13 phage receptor. The pIII-STE fusion was affinity purified and its ability to bind irreversibly to the F pilus tip and effectively block M13KO7 infection was demonstrated.

Radioactively labelled F pili were purified using the pOX38-*tra715* plasmid expression system (Maneewannakul *et al.*, 1992). An affinity purification procedure to enrich for the putative pilus tip protein was developed using immobilized pIII-STE fusion and radioactively labelled pili. Analysis of the products from the affinity purification failed to identify any minor pilus-associated proteins with affinity for pIII-STE. However, increased

Fig. 4.7 Analysis of pIII-STE-pilus tip interactions by affinity purification using biotinylated acrylic beads. Affinity purification to isolate the putative pilus tip protein was performed using sheared radioactively labelled pili and pIII-STE pre-adsorbed to biotinylated acrylic beads as described in Section 4.4. After removing the unbound material, the beads were successively washed with PBS pH 7.3 containing 0.5 M NaCl. The beads were then resuspended in SDS sample buffer and were heated to 100°C to elute the material bound to pIII-STE. The unbound, wash and eluate fractions as well as total cellular proteins from XK100 cells harbouring pOX38-*tra715* were separated on a 15% SDS-polyacrylamide gel. The proteins were transferred to a PVDF membrane and the membrane was processed by 'Far Western' analysis to detect proteins with affinity for pIII-STE as described for Fig. 4.6A. The immunoblot from the 'Far Western' analysis is shown in **A** and the autoradiogram of the dried membrane is shown in **B**. Lane 1: total cellular proteins from pOX38-*tra715*/XK100 cells, lane 2: unbound fraction, lanes 3-5: wash fractions and lane 6: eluate. The positions of the 29 kDa band in **A** is indicated. The molecular mass markers in kDa are shown on the left.


1
2
3
4
5
6
68
43
29
18.4
14.3
6.3
A
B

amounts of pilin was seen in the eluate fractions indicating an association between pilin and pIII-STE.

'Far Western' analysis further identified a 29 kDa host-encoded protein of an unknown nature which exhibits an affinity for pIII-STE. The only *E. coli* protein that has been shown to interact with pIII is TolA, a 44 kDa periplasmic protein of the *tolQRA* gene cluster whose products are essential for M13 phage entry (Section 1.6.2; Riechmann and Holliger, 1997; Click and Webster, 1997). Recently, however, Vianney *et al.* (1996) identified a new open reading frame in the *tol* gene cluster designated *orf2* which encodes a 29 kDa periplasmic protein. Although insertion mutations in *orf2* do not prevent M13 infection, the possibility that the product of *orf2* corresponds to the 29 kDa band identified in the 'Far Western' analysis is currently under investigation.

The demonstrated ability of pIII-STE fusion protein to attach to the F pilus tip with high affinity and specificity proves that it is by far the best ligand to isolate the putative tip protein. However, failure to identify a radioactively labelled transfer protein that binds pIII-STE suggests that a unique tip protein may not exist. In support of this notion, prior examination of purified pili by mass spectroscopy, Western blot analysis with polyclonal pilus-specific antiserum and by radioactive labelling also failed to identify a minor protein. Also, taking into account the subunit mass per length of 3 000 Da per Å calculated for F pili (Folkhard *et al*, 1979) and the molecular weight of 7 200 for F pilin (Frost *et al.*, 1984), a pilus of an average length of 1.5 μm would contain approximately 6 249 pilin monomers. Assuming a ratio of 1:6 249 of tip protein to pilin per pilus filament, 1 μg of purified pili should contain approximately 0.16 ng of tip protein if the molecular weights of pilin and the tip protein were similar (the molecular weights of all the pilus assembly proteins are higher than that of mature pilin). Since picogram amounts of proteins can be detected with immunological methods and by autoradiography (Ausubel *et at.*, 1989), this amount should be visible on Western blots and in radioactively labelled pili.

Thus, the M13 phage receptor is most likely made up of a unique configuration of pilin subunits at the tip. Since the subunits in both F pili and filamentous bacteriophages are related by a five-fold rotational symmetry, Marvin and Folkhard (1986) had suggested that

phage adsorption occurs by a specific tip-to-tip symmetry match mediated by five pIII proteins. However, as shown by the simultaneous binding of 1-3 pIII-STE fusion proteins (Fig. 4.3) or a maximum of three phage particles (Marvin and Hohn, 1969) to the pilus tip, pilus-phage interaction could be mediated by attachment of one pIII molecule to a region on pilin exposed at the tip rather than by a perfect symmetry match.

Pilin residues which define the attachment domain have never been identified due to the absence of mutations that specifically alter phage attachment. These residues could also be required for important aspects of pilus assembly such as subunit-subunit interactions. Since residues that are involved in protein-protein interactions are often conserved, the hexapeptide $V_{16}KATFG_{21}$ found in F, ColB2, R100-1 and pED208 pilin proteins has been implicated in phage attachment (Section 1.4.5; Ippen-Ihler and Minkley, 1986). If the hexapeptide forms the binding domain, lack of interactions between pIII-STE fusion and pilin in 'Far Western' analysis (Fig 4.6A), could be attributed to the inaccessibility of this domain in unassembled pilin. Alternatively, the attachment domain could be exposed in a unique structural conformation in a tip-specific manner. To ascertain the role of the hexapeptide in phage attachment, future studies should address the cloning and sequencing of the pilin gene from the IncD plasmid, R711b, whose pili also attach M13 phage (Bradley and Meynell, 1978). If a conserved sequence is also identified, synthetic peptides carrying this sequence could be synthesized and tested for their ability to inhibit M13KO7 infection. Subsequently, monoclonal antibodies raised against this peptide could be tested for binding to the F pilus tip by immunogold electron microscopy.

4.4 MATERIALS AND METHODS

4.4.1 Bacterial strains, plasmids and growth conditions

The *Escherichia coli* K-12 strains used in this study were K802 (*supE hsdR gal metB*, Wood, 1966), DH5α {(*Ø80d lacZΔM15) Δ(lacZ2A-argF) U169 deoR recA1 endA1 hsdR17* (r_K^-, m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1*; Ausubel *et al.*, 1989} and DH5αF' (DH5α harbouring the F plasmid, Ausubel *et al.*, 1989). The *E. coli* B strain, XK100, is a spontaneous

spectinomycin^R derivative of BL21 (DE3) which carries a *lacUV5*-regulated T7 RNA polymerase gene on its chromosome [F*ompT hsdS*$_B$(r_B m_{BB})*gal dcm* (DE3); Maneewannakul, *et al.*, 1991]. The plasmids, pIII-STE and pOX38-*tra715*, are described in Section 4.1. The plasmid, pDMI.1, carries a p15A ori and overexpresses the *lac* repressor (Lanzer, 1988). Bacteria were grown either in liquid or on solid LB medium (Sambrook *et al.*, 1989) with appropriate concentrations of antibiotics.

4.4.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The procedures for SDS-PAGE are essentially as described in Sambrook *et al.* (1989) with slight modifications. For all applications in this study, 10% or 15% SDS-polyacrylamide gels were used. The composition for a 0.75 mm thick 15% separating gel was: 10.5 ml of 30:0.8 acrylamide:bis-acrylamide mixture; 4.6 ml 0.4% NaCl; 5 ml Tris-HCl pH 8.8; 0.2 ml 10% SDS; 0.2 ml 10% APS and 0.008 ml TEMED. The composition for a 7% stacking gel was: 2.3 ml 30:0.8 acrylamide:bis-acrylamide mixture; 6.1 ml of MilliQ-H_2O; 1.25 ml Tris-HCl pH 6.8; 0.1 ml 10% SDS; 0.1 ml 10% APS and 0.01 ml TEMED.

For analysis of cell lysates, approximately $1x10^7$ cells from a culture in the exponential growth phase (OD_{600} of 0.8-1.0) were centrifuged for 5 min at 2000 g at 4°C. The cell pellets were solubilized in SDS sample buffer (50 mM Tris-HCl pH 6.8; 2% SDS; 10% glycerol; 100 mM DTT and 0.1% bromophenol blue) to yield a preparation of total cellular proteins. For analysis of proteins in liquid suspension, the samples were dried under vacuum and resuspended in SDS sample buffer. Along with protein molecular weight standards (GIBCO BRL) all the samples were heated at 100°C for 5 min prior to SDS-PAGE. Electrophoresis was carried out in Laemmli running buffer (25 mM Tris.HCl, 250 mM glycine and 0.1% SDS; Laemmli, 1970) at 35 mA for 2.5 hours. All reagents used were of highest purity electrophoretic grade. The proteins were visualized by either staining the gel with silver nitrate or by Western blot analysis.

4.4.3 Visualizing proteins by staining with silver nitrate

The procedure used for silver staining was adapted from Oakley *et al.* (1980). After electrophoresis, the SDS-polyacrylamide gel was fixed overnight in 200 ml of freshly prepared Fixing Solution I (50% methanol and 12% glacial acetic acid). The gel was washed twice in MilliQ-H_2O for 20 min each, and was soaked in 200 ml of freshly prepared Fixing Solution II (1.9% $Na_2B_4O_7$ and 4% glutaraldehyde) for 15 min. Following repeated washings in MilliQ-H_2O (3x 20 min), the gel was stained for 20 min in 200 ml of Staining Solution (18.5 mM NaOH; 1.96% NH_4OH and 0.8% $AgNO_3$). After removing the stain by washing in MilliQ-H_2O for a maximum of 15 min, the gel was soaked in 200 ml of Developing Solution (10% ethanol; 0.006% citric acid and 0.01% formaldehyde) until protein bands were visible (2-30 min). The reaction was halted by rinsing the gel with copious amounts of MilliQ-H_2O, and when necessary it was destained in Destaining Solution [0.2% $Na_2S_2O_3$ and 0.01% ($K_3Fe(CN)_6$)] to reduce the background.

4.4.4 Western Blot Analysis

Immediately following electrophoresis, the SDS-polyacrylamide gel was pre-equilibrated in Bjerrum and Schafer-Nielson transfer buffer (48 mM Tris.HCl, 39 mM glycine, 0.04% SDS and 20% methanol, pH 9.2; Protein Blotting: A guide to transfer and detection, 1992, Bio-Rad Laboratories). Proteins were blotted on a sheet of pre-equilibrated Immobilon PVDF membrane (polyvinylidene difluoride, Millipore) using a Trans-Blot electrophoretic transfer cell (Bio-Rad) at 150 mA for 3 hours at 4°C as described by Towbin *et al.* (1979).

The membrane was briefly rinsed in Tris-buffered saline pH 7.5 (TBS: 100 mM Tris.HCl, 0.9% NaCl, Ausubel *et al.*, 1989) and blocked in Blocking solution (10% skim milk, 0.01% Tween-20 in TBS pH 7.5) at 4°C overnight. The membrane was rinsed briefly in TBST (TBS + 0.01% Tween-20) and was incubated with rabbit anti-streptavidin primary antiserum diluted 1:5 000 in Blocking solution for 1 hour at RT. Excess primary antibody was removed by repeated washings in TBST for a total of 30 min. The secondary antibody, anti-rabbit IgG coupled to Horseradish peroxidase (HRP, Amersham Life Science), was diluted 1:2 000 in Blocking solution and the membrane was incubated in the mixture for 1

hour at RT. After repeated washings in TBST to remove the unbound antibodies, the membrane was placed on a sheet of acetate transparency. The peroxidase substrate-enhancer mixture (0.5 ml; provided in the chemiluminescence kit, Boehringer Mannheim) was pipetted on the membrane and allowed to spread evenly by tightly placing a second sheet of transparency on top of the membrane. After 1 min, the membrane was wrapped in Saran Wrap and was exposed to a Kodak X-Ray film for various lengths of time (30 sec-30 min) until the desired level of band intensity was achieved.

4.4.5 'Far Western' Analysis

'Far Western' analysis (Phizicky and Fields, 1995) was carried out as described for the Western blotting procedure with the following additional steps. After the initial blocking step, the membrane was incubated with 10 pmol of pIII-STE fusion in blocking solution for 1 hour at RT. Following washings in TBST, the membrane was incubated with primary and secondary antisera.

4.4.6 Expression and purification of soluble pIII-STE fusion protein

Escherichia coli K802 cells were transformed with plasmids pIII-STE and pDMI.1. Transformants were selected on LB medium containing 50 µg/ml ampicillin, 25 µg/ml kanamycin and 100 mM glucose. Routinely, ten colonies were screened by Western blotting for the production of pIII-STE fusion after induction with IPTG as described in subsequent sections.

For large scale purification, a single colony producing the protein was inoculated into 5 ml LB broth containing ampicillin (50 µg/ml), kanamycin (25 µg/ml) and 100 mM glucose and was grown at 30°C overnight. Two ml of this inoculum were used as a seed culture to inoculate a 200 ml culture which was grown at 30°C and 260 rpm until OD_{600} 0.8-1.0 was reached. IPTG (Sigma Chemicals) was added to a final concentration of 100 µM to induce the expression of pIII-STE and growth was continued at RT for a further 3 hours. Cells were harvested by centrifugation at 2000 g for 15 min at 4°C. Periplasmic fractions were prepared by subjecting the cells to osmotic shock as described below (Ausubel *et al.*, 1989). The cell

pellet was immediately resuspended in 20 ml of Solution A [50 mM Tris.HCl pH 8.0, 20% sucrose (w/v) and 1 mM EDTA] and left on ice for 20 min with occasional shaking. After centrifugation at 12 100 g for 10 min at 4°C, the cell pellet was resuspended in 20 ml of Solution B (5 mM $MgSO_4$) and was incubated on ice as described above. The supernatant representing the osmotic shock fluid rich in periplasmic proteins was collected by centrifugation and was used for affinity purification in the following steps. To remove any remaining particulate material, the supernatant was re-centrifuged at 30 000 rpm in SW 50.1 Ti rotor for 30 min at 4°C and was dialyzed against PBS pH 7.3 (150 mM NaCl, 50 mM $NaH_2PO_4.H_2O$, Ausubel *et al.*, 1989) at 4°C overnight. In preparation for affinity purification, 1 ml of precharged nickel-nitrilotriacetate (Ni^{2+}-NTA) sepharose resin (a gift from Dr. J. Elliot, Dept. of Medical Microbiology and Immunology, U. of Alberta) was washed 3 times with 10 volumes of MilliQ-H_2O and was pre-equilibrated in 5 volumes of Buffer A (1 M NaCl, 50 mM $NaH_2PO_4.H_2O$ and 10 mM imidazole pH 7.5) at 4°C overnight. Excess buffer was removed by centrifugation at 12 100 g for 15 min. The composition of the dialyzed supernatant was adjusted to that of Buffer A and it was mixed with the pre-equilibrated Ni^{2+}-NTA resin. Binding was done at 4°C overnight with gentle agitation. The unbound material was removed by centrifugation at 12 100 g for 15 min at 4°C and the resin was washed successively with 10 volumes of Buffer A and Buffer B (1 M NaCl, 50 mM $NaH_2PO_4.H_2O$ and 30 mM imidazole pH 7.5) for 15 min each at 4°C. The adsorbed pIII-STE fusion protein was first eluted with 5 volumes of Buffer C (1 M NaCl, 50 mM Na_2PO_4 and 400 mM imidazole pH 7.5) then with 5 volumes of Buffer D (50 mM EDTA in PBS pH 6.5). The unbound, wash and eluate fractions were dialyzed extensively against PBS pH 7.3. The eluate fractions were stored at -80°C as 1 ml aliquots in siliconized Eppendorf tubes in the presence of 1 mM protease inhibitor, Pefabloc®SC (Boehringer Mannheim). One hundred µl aliquots of each fraction were dialyzed against MilliQ-H_2O, dried under vacuum and separated on a 10% SDS-polyacrylamide gel. The pIII-STE fusion protein was detected on Western blots with rabbit anti-streptavidin antiserum (Sigma Chemicals) and was quantified using known amounts of streptavidin (Sigma Chemicals) as the standard.

4.4.7 Assay for inhibition of M13KO7 infection of F' cells by pIII-STE fusion

E. coli DH5α or DH5αF' cells were grown in LB medium to mid-logarithmic phase. Approximately $1x10^7$ cells were mixed with either 5 pmol of pIII-STE fusion or approximately the same amount of bovine serum albumin (BSA, Boehringer Mannheim) as the negative control. Following incubation on ice for 30 min, $1x10^7$ pfu of M13KO7 phage suspension (MOI=1; Vieira and Messing, 1987) was added to the mixture. After continued incubation for an additional 10 min, the mixture was transferred to 37°C for 5 min to allow phage entry. Unattached phage particles were removed by centrifugation at 2000 g for 5 min at RT. The cell pellet was washed twice with LB to dislodge any remaining phage particles then resuspended in 500 µl of LB and continued incubation at 37°C for 30 min to allow phage replication and multiplication to occur. Serial dilutions were plated on LB agar with and without 70 µg/ml kanamycin to determine the total number of cells and the number of infected cells, respectively. The percent infection was determined as the ratio of Km^R colonies to the total number cells.

4.4.8 Demonstration of pIII-STE binding to the F pilus tip by immunogold electron microscopy

Approximately 5 ng of pIII-STE fusion was added to 100 µl of DH5αF' cells grown to logarithmic phase. Using a wide-bore pipette tip the mixture was gently mixed and was incubated on ice for 1 hour. Twenty µl of the mixture was placed on a Formvar-coated, carbon-shadowed copper grid using a wide-bore pipette tip and was left to adsorb for 3 min. Unadsorbed sample was removed by blotting the edge of the grid with a Whatman filter paper (Maher *et al.*, 1993). Free binding sites were blocked by initially placing the grid on a droplet of bovine fetal clone (HyClone Lab. Inc., Utah, USA) for 5 min then on fresh droplets of Blocking solution (1% BSA in PBS pH 7.3) for a total of 15 min. Binding of the primary antibody was done by placing the grid on a droplet of rabbit anti-streptavidin antiserum diluted 1:5000 in Blocking solution for 60 min. After repeated washings in Blocking solution as described above, the grid was incubated for 30 min on a droplet of the secondary antibody, anti-rabbit IgG conjugated to colloidal gold (10 nm gold particles; EY

Labs., California, USA) diluted 1:100 in Blocking solution. After repeated washings in distilled deionized water for a total of 20 min, the grid was floated on a droplet of R17 phage suspension for 30 min to label the F pili. Following a brief final wash in distilled deionized water, the grid was negatively stained with 1% phosphotungstic acid pH 7.0, and was examined with a Philips model 410 transmission electron microscope.

4.4.9 Purification of [^{35}S]-methionine-labelled F pili

Radioactively labelled F pili were purified using the pOX38-*tra715* plasmid expression system in XK100 host cells. A 2 ml overnight culture of pOX38-*tra715* /XK100 cells was used to inoculate 500 ml LB containing 25 µg/ml kanamycin. The culture was grown with aeration at 37°C until OD_{600} of 0.8-1.0 was reached. The cells were pelleted by centrifugation at 2000 g for 10 min at RT, washed once with an equal volume of M9 medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl/1l H_2O pH 7.4; Sambrook *et al.*, 1989), then resuspended in 500 ml of ^{35}[S]-methionine-assay medium (2 mM $MgSO_4$, 0.2% glucose, 0.1 mM $CaCl_2$, 0.1% 18-aa mixture, in M9 medium; Ausubel *et al.*, 1989). Growth was resumed at 37°C for 1 hour. IPTG was added to a final concentration of 10 mM to induce the expression of chromosomally encoded T7 RNA polymerase and growth was continued for 1 hour. Following the addition of 200 µg/ml rifampicin (Sigma Chemicals) to halt host cellular transcription, the culture was shifted to 42°C for 10 min to facilitate the uptake of rifampicin (Ausubel *et al.*, 1989) then transferred to 37°C for an additional 40 min. Two hundred µCi of ^{35}[S]-methionine (Mandel Scientific, NEN™ Life Science Products) was added and protein labelling was allowed to proceed for 10 min.

The culture was passed through a 50 ml syringe fitted with a 26-gauge needle in order to shear the remaining cell-associated pili. After removing the cells by centrifugation at 12 100 g for 15 min at 4°C, pili in the supernatant were precipitated overnight at 4°C with 2% polyethylene glycol 8000 (PEG) and 0.5 M NaCl (Armstrong *et al.*, 1980). The precipitate was collected by centrifugation at 12 100 g for 30 min at 4°C, resuspended in 5 ml of MilliQ-H_2O and was dialyzed extensively against MilliQ-H_2O to remove the PEG. The dialyzed pili suspension was dried under vacuum and the pellet was redissolved in 0.4 ml of SSC buffer

(0.15 M NaCl and 0.015 M sodium citrate, pH 7.0; Armstrong *et al.*, 1980) and was layered on a CsCl density gradient (ρ=1.30 g/cm^3) in a thin-wall polycarbonate ultracentrifuge tube. The gradient was run at 35 000 rpm in SW 50.1 Ti rotor for 24 hours at 5°C. At the end of the run, two bands were visible. Two hundred µl fractions from the gradient was collected and the amount of radioactivity in each fraction was determined by counting 3 µl from each fraction in a scintillation counter. Five µl aliquots from the fractions containing significant amount of radioactivity were separated on a 15% SDS-polyacrylamide gel. Pili were visualized by autoradiography of the dried gel. The fractions containing pili were dialyzed against MilliQ-H_2O prior to storage at 4°C. The refractive index of the fraction containing pili was determined by spotting 5 µl of the suspension on a refractometer. An estimate of the amount of purified pili was determined with reference to known amounts of protein standards (Gibco BRL) on a 15% SDS-polyacrylamide gel stained with silver nitrate.

4.4.10 Affinity purification of the putative pilus tip protein with pIII-STE immobilized on Ni^{2+}-NTA resin

Approximately 0.75 µg of [^{35}S]-methionine labelled pili were combined with 1 ml of PBS pH 7.3 and the mixture was sonicated for 10 min as described in Section 7.4. The sonicated pili were added to 100 ng of pIII-STE fusion preadsorbed on 300 µl of Ni^{2+}-NTA resin. Binding was done with gentle rocking at 4°C overnight. A negative control in which the pili were mixed with 300 µl of Ni^{2+}-NTA resin without the adsorbed fusion protein was carried through the same procedures. Washing and elution steps were done as described previously for the purification of pIII-STE fusion (Section 4.4.6) except the volume used at each step was 2 ml. The unbound, wash and eluate fractions were dialyzed against MilliQ-H_2O, dried under vacuum, divided into half and separated on two 15% SDS-polyacrylamide gels. One gel was stained with silver nitrate while the other was processed by 'Far Western' analysis as described in Section 4.4.5.

4.4.11 Affinity purification of the putative pilus tip protein with pIII-STE fusion immobilized on biotinylated acrylic beads

Approximately 1 mg of biotinylated acrylic beads (Sigma Chemicals) was washed several times in MilliQ-H_2O then pre-equilibrated in Binding buffer (PBS pH 7.3 containing 500 mM NaCl) overnight. One hundred ng of pIII-STE fusion was adsorbed to the beads for 4-5 hours at 4°C. The beads were recovered by centrifugation at 12 100 g and 0.75 µg of sonicated pili was mixed with the beads in 1 ml of Binding buffer. Following binding at 4°C overnight, the unbound material was removed by centrifugation and the beads were washed repeatedly with 5 ml of Binding buffer. Unbound and wash fractions were dialyzed against MilliQ-H_2O, dried under vacuum and resuspended in SDS sample buffer. Forty µl of SDS sample buffer was added to the beads and all the samples were heated at 100°C for 5 min prior to SDS-PAGE.

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CHAPTER 5

Analysis of allelic specificity of pilus synthesis genes and characterization of pili produced by an F *traX* mutant

5.1 INTRODUCTION

The broad aim of the work presented in this chapter is directed at exploring whether or not a genetic basis exists for the phenotypic variation between F and R100-1 pili, namely in their ability to confer sensitivity to pilus-specific phages and surface exclusion specificity. This was undertaken by constructing a series of chimeric plasmids carrying segments of R100-1 *tra* DNA encoding the fifteen genes involved in pilus synthesis. These chimeras aided in the determination of the nucleotide sequence of R100-1 pilus synthesis genes and facilitated subsequent comparison of their gene products with those of F in order to catalogue the differences in their primary sequences that might contribute to their specificity of function, if any. In addition, complementation analyses using a series of F plasmid derivatives carrying defined mutations in each of the fifteen genes with the corresponding gene from R100-1 permitted an assessment of the contribution of each of the pilus synthesis genes in altering sensitivity to pilus-specific phage infection and surface exclusion specificity. Only the findings of the complementation studies are presented in this chapter and the results are discussed with respect to the ability of each of these genes to alter specificity in pilus function.

The *traX* gene product which is required for the N-terminal acetylation of pilin has been of particular interest since despite the conservation of N^{α}-acetylation in most F-like pilin, this feature is dispensable to pilus assembly and function (Section 1.4.2). Several reports however, have suggested that pili composed of unacetylated subunits are structurally different than wild-type pili (Grossman *et al.*, 1990, Silverman, 1997). This is based on the observation that F pili synthesized by the plasmid pTG801, a pUC plasmid derivative expressing all the pilus synthesis genes except *traX* under the control of the *lac* promoter (Section 1.4.2), laterally attached the monoclonal antibody (mAb), JEL92 (Section 1.4.4), whose epitope near the N-terminus is normally masked in wild-type pili (Section 1.4.3). To ascertain if post-translational modification of pilin alters the subunit conformation in the pilus filament, the serological properties of pili synthesized by an F *traX* mutant was tested by immunogold electron microscopy.

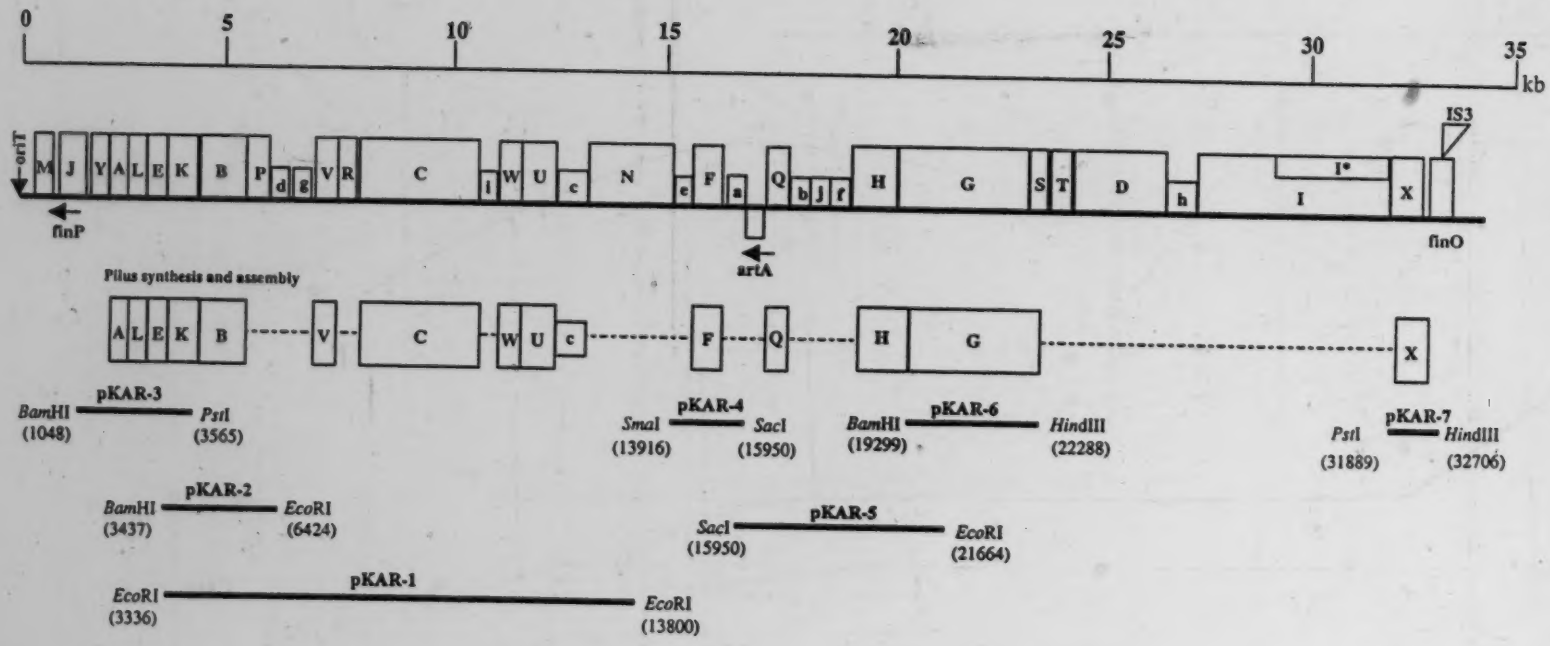
Further, Firth and Skurray (1995) have suggested that TraX belongs to a new protein family involved in post-translational modification of subunits associated with the assembly of conjugative and type IV pili. They further proposed that acetylation of cognate subunits might provide structural variation in conjugative pili which might affect sensitivity to pilus-specific phages. To explore this possibility further, the ability of R100-1 TraX to acetylate F pilin was investigated in order to determine if the allelic variation between F and R100-1 *traX* reflects acetylation specific for their cognate pilin proteins.

5.2 RESULTS

5.2.1 Construction of recombinant plasmids expressing the pilus synthesis genes from R100-1

Based on the physical map of R100 (NR1) reported by Womble and Rownd (1988), chimeric plasmids encoding various R100-1 pilus synthesis genes were constructed in order to facilitate the nucleotide sequence determination of its *tra* region as well as to perform complementation analyses described in the following section. The *tra* segment carried by the plasmids pKAR-1 to pKAR-7 together with the corresponding coordinates in the F *tra* map (Frost *et al.*, 1994), and a description of each of the constructs are presented in Fig. 5.1 and Table 5.4, respectively. The plasmids pKAR-1, pKAR-3, pKAR-4 and pKAR-5 were constructed by 'shotgun' cloning of DNA fragments obtained from restriction digestion of R100-1 plasmid DNA with appropriate restriction enzymes, and subsequent cloning into either the medium copy number or the low copy number vectors, pT7-4 and pACYC177, respectively (Table 5.4). Recombinant clones were identified by colony hybridization of the resulting transformants with [^{32}P] end-labelled olignucleotide probes as described in Section 5.4. Together, these four chimeras encode the pilus synthesis genes *traA, L, E, K, B, V, C, W, U, F, Q, H and trbC.* DNA fragments encoding the *traG* and *traX* genes in isolation from other *tra* cistrons were obtained by PCR amplification and subsequent cloning into the pT7-3 vector to generate the plasmids, pKAR-6 and pKAR-7, respectively. The vectors, pT7 and pACYC177 (Table 5.4), were chosen for the construction of the pKAR chimeras due to their

Fig. 5.1 Diagram of transfer genes expressed by various pKAR plasmids. The top line gives the length in kilobases (kb) of the F transfer region. The second line represents all the *tra* (uppercase) and *trb* (lowercase) genes, *artA* and *finP* are transcripts in the anti-*tra* orientation, *oriT* is the origin of transfer, IS*3* refers to the insertion element in *finO*, and I* is a gene product encoded within the *traI* gene. The third line represents the gene products involved in pilus synthesis and assembly. This figure was adapted from Frost *et al.*, 1994. The corresponding F *tra* region encoded by each of the chimeras is shown as a black line together with the F coordinates and the restriction sites flanking the inserts. With the exception of the *Sac*I site at nucleotide 15950, none of the sites is present in the F map. The actual size of the insert in pKAR-1 is 11.6 kb due to the presence of additional open reading frames in the R100-1 sequence.


0
5
10
15
20
25
30
35
kb
oriT
M J Y A L E K B P d g V R C I W U c N e F a Q b j f H G S T D h I I* X
IS3
finP
artA
finO
Pilus synthesis and assembly
A L E K B V C W U c F Q H G X
pKAR-3
BamHI (1048)
PstI (3565)
pKAR-4
SmaI (13916)
SacI (15950)
pKAR-6
BamHI (19299)
HindIII (22288)
pKAR-7
PstI (31889)
HindIII (32706)
pKAR-2
BamHI (3437)
EcoRI (6424)
pKAR-5
SacI (15950)
EcoRI (21664)
pKAR-1
EcoRI (3336)
EcoRI (13800)

low copy numbers to avoid complications arising from overexpression of *tra* proteins. The orientation of the inserts in pKAR-3, pKAR-4 and pKAR-5 constructs were such that the transcription of the *tra* genes was under the control of either the ß-lactamase or kanamycin gene promoters of the vector. Whereas the expression of *traG* and *traX* in pKAR-6 and pKAR-7 respectively, relied on transcription read-through originating from the ß-lactamase promoter of the vector, the expression of *tra* genes encoded by pKAR-1 in which the insert is in the reverse orientation to the ß-lactamase gene, is believed to be due to the presence of internal promoters within the *tra* region. All of the pKAR constructs were stably maintained in the cells and expressed *tra* proteins at levels adequate for complementation analysis of F *tra* mutant derivatives as described in the following section.

pKAR-2 which was constructed for the determination of nucleotide sequence of *traK, B* and *P* genes previously described in Chapter 3 (Table 3.4), is included in Fig. 5.1 simply as a reference and it was not used in the complementation studies due to its high copy number (pUC118 derivative). The nucleotide sequences of all the pilus synthesis genes encoded by pKAR chimeras have been determined and will be reported elsewhere.

5.2.2 Complementation of F *tra* mutants for donor conjugal transfer and pilus-specific phage infection by pKAR chimeras

A series of derivatives of the F plasmid carrying mutations in each of the pilus synthesis genes are listed in Table 5.3. With the exception of pOX38-*traX482*, all the other mutants are deficient in pilus production. To ascertain whether the pKAR plasmids were able to complement these mutations and restore functional pilus synthesis, *E. coli* host cells harbouring these mutants were transformed with one of the pKAR plasmids and the efficiency of plasmid transfer was measured in a mating assay (Section 2.4) with *E. coli* ED24 as the recipient cells. For comparison, chimeric plasmids carrying the equivalent segments of the F *tra* operon (Table 5.4) were also tested for their ability to complement these mutations. The efficiency of DNA transfer, expressed as the number of transconjugants per 100 donors both in the presence and absence of complementing plasmids, is listed in Table 5.1. Complementation analysis of pOX38A::CAT was omitted here since the results have been

Table 5.1 Transfer proficiency and phage sensitivity profiles of derivatives of F *tra* and *trb* mutants expressing the corresponding genes from F and R100-1 plasmids

tra mutant +/- complementing plasmid	[c] Mating efficiency (%) transconjugants/100 donors	[a] Phage sensitivity f1	R17
F*lac traL311*	0.01	R	R
F*lac traL311* + pLF164	83.33	S	S
F*lac traL311* + pLF107	67.77	S	S
F*lac traE18*	0.00	R	R
F*lac traE18* + pRS27	3.13	S	S
F*lac traE18* + pKAR-3[b]	0.14	U	U
F*lac traK105*	0.00	R	R
F*lac traK105* + pRS27[b]	0.005	U	U
F*lac traK105* + pKAR-1[b]	0.25	U	U
F*lac traB2*	0.00	R	R
F*lac traB2* + pRS27	4.50	S	S
F*lac traB2* + pKAR-1	2.40	S	S
F*lac traV569*	0.02	R	R
F*lac traV569* + pKI164	16.67	S	S
F*lac traV569* + pKAR-1	0.85	S	S
F*lac traC5*	0.008	R	R
F*lac traC5* + pRS29	0.80	S	S
F*lac traC5* + pKAR-1	16.67	S	S
F*lac traW546*	0.02	R	R
F*lac traW546* + pRS29	40.00	S	S
F*lac traW546* + pKAR-1	20.00	S	S

Table 5.1 Cont.

pOX38-*traU347*	0.00	R*	R*
pOX38-*traU347*+ pRS29	12.50	S	S
pOX38-*traU347*+ pKAR-1	38.46	S	S
pOX38-*trbC460*	0.00	R	R
pOX38-*trbC460* + pRS29	0.71	S	S
pOX38-*trbC460* + pKAR-1	21.43	S	S
F*lac traF13*	0.00	R	R
F*lac traF13* + pKI175	100.00	S	S
F*lac traF13* + pKAR-4	100.00	S	S
pOX38-*traQ238*	0.003	R	R
pOX38-*traQ238* + pRS29	6.35	S	S
pOX38-*traQ238* + pKAR-5	46.17	S	S
F*lac traH80*	0.00	R	R
F*lac traH80* + pRS29	0.095	S	S
F*lac traH80* + pKAR-5	13.65	S	S
F*lac traG106*	0.00	R	R
F*lac traG106* + pRS1670	36.12	S	S
F*lac traG106*+ pKAR-6	25.85	S	S
pOX38-*traX482*	67.00	S	S
pOX38-*traX482* + pKAF-3	100.00	S	S
pOX38-*traX482* + pKAR-7	100.00	S	S
pOX38-Km	67.34	S	S
R100-1	97.20	R*	R

[a] Phage sensitivity was determined by qualitative spot tests (Section 2.4). S represents 70-100% of the sensitivity exhibited pOX38-Km. R is resistant; R* is weakly sensitive (approximately 1%); U is undetermined

[b] The phage sensitivity profiles for F*lac traE18* + pKAR-3, F*lac traK105* + pKAR-1 and F*lac traK105* + pRS27 could not be discerned from spot tests due to low levels of complementation. However, pili from these cells when examined by EM attached both R17 and f1 phages.

[c] All F*lac* mutants were in *E. coli* ED2149, all pOX38 derivatives were in *E. coli* XK1200 while R100-1 was in *E. coli* JE-1. The recipient for matings was ED24 while for R100-1, ED24 carrying the pT7-3 vector was the recipient.

discussed previously in Chapter 2. With the exception of the pOX38-*traX482* mutation which does not compromise DNA transfer ability, the pKAR plasmids were able to restore donor conjugal activity at levels comparable to or higher than their F counterparts; however, the complementation levels of F*lac traE18* and F*lac traV569* mutations were approximately 20% lower. Unusually low levels of transfer were observed for F*lac traK105* in the presence of either pRS27 or pKAR-1 plasmids. Since *traK105* is a frameshift mutation, reduction in the observed complementation levels could be due to the presence of a non-functional *traK* product which might compete with the functional TraK supplied in trans.

Since the pKAR chimeras were able to complement F *tra* mutations for DNA transfer, the pili produced in the presence of these chimeras were tested for their ability to confer sensitivity to pilus-specific phages. Susceptibility to both f1 and R17 infection for each of the F *tra* mutants was determined using qualitative phage spot tests (Section 2.4) and the results are presented in Table 5.1. Except for F*lac traE18* and F*lac traK105*, the pKAR plasmids were able to restore full sensitivity to both phages when co-resident with any one of the F *tra* mutants. Although lysis could not be observed on spot tests for both F*lac traE18* complemented with pKAR-3 and F*lac traK105* complemented with pKAR-1 due the relatively few pili present, these pili attached both phages well when examined by TEM (data not shown). These results indicate that the R100-1 pilus synthesis genes complement the corresponding mutation in the F plasmid to produce functional pili which are phenotypically F-like in their ability to confer susceptibility to pilus-specific phage infection.

5.2.3 Analysis of surface exclusion properties of F *tra* mutants expressing the corresponding pilus synthesis genes from F and R100-1 plasmids

In order to determine if the surface exclusion specificity between F and R100-1 plasmids is due to the allelic differences of one or more pilus synthesis genes, the mating efficiency of F *tra* mutants complemented with either the corresponding F or R100-1 genes described in the previous section, to recipient cells expressing the F surface exclusion proteins were measured. Mating assays were performed with recipient *E. coli* ED24 harbouring either the pSC101 vector control or the plasmid pRS31 which expresses both F TraT and TraS proteins (Table

5.4). Despite the high levels of synthesis of both surface exclusion proteins by pRS31, this plasmid is stably maintained in the cell and its presence has been reported to reduce the entry of the F plasmid approximately 10 000-fold (Achtman *et al.*, 1977). The surface exclusion (Sfx) index which is a measure of reduction in plasmid transfer (Section 2.4), for each of the F *tra* mutants as well as pOX38-Km and R100-1 plasmids, is given in Table 5.2. As expected, the presence of pRS31 in the recipient cell reduces the entry of pOX38-Km plasmid approximately 1000-fold with a Sfx index of 1069.77 when compared to the R100-1 plasmid whose Sfx index is only 11.61. Although these values are lower than those reported by Achtman *et al.* (1977), they are nevertheless consistent with the surface exclusion specificity of each plasmid. The Sfx indices for *tra* mutants expressing either F or R100-1 pilus synthesis genes range from 346.67 for F*lac traK105* complemented with pRS27 to 5787.81 for F*lac traG106* complemented with pRS29. Although the Sfx indices as a whole fall over a wide range, the Sfx values for *tra* mutants complemented with R100-1 genes do not differ significantly from the values obtained when complemented with F genes, with *traG* being the only exception. The transfer of F *lac traG106* expressing R100-1 *traG* was approximately 600-fold higher than F *lac traG106* expressing F *traG*. Since none of the other fourteen R100-1 pilus synthesis genes drastically reduced the Sfx indices, it is evident that *traG* determines the surface exclusion specificity.

5.2.4 R100-1 TraX restores N-terminal acetylation of F pilin in pOX38-*traX482*

Since the pOX38-*traX482* mutation which prevents the N-terminal acetylation of F pilin bears a wild-type phenotype, the ability of R100-1 *traX* to complement this mutation was determined by testing for acetylation of pilin subunits. Total cellular proteins from *E. coli* MC4100 cells harbouring the plasmid pOX38-*traX482* in the presence or absence of either pKAF-3 (F *traX*) or pKAR-7 (R100-1 *traX*) were fractionated by SDS-PAGE (Section 4.4). Pilin polypeptides were detected immunologically with monoclonal antibodies JEL92 and JEL93 on Western blots (Section 4.4). The mAb JEL92 recognizes an internal amino acid sequence in F pilin and thus is reactive with both acetylated and unacetylated pilin. JEL93,

Table 5.2 Surface exclusion properties of F *tra* and *trb* mutant derivatives expressing the corresponding genes from F and R100-1 plasmids

tra mutant + complementing plasmid	[a,b,c] Mating efficiency (%) transconjugants/100 donors		[b] Surface Exclusion Index
	pSC101	pRS31	
pOX38-Km	51.67	4.83×10^{-2}	1069.77
R100-1	24.61	2.12	11.61
pOX38A::CAT + pLF164	4.90	6.70×10^{-3}	731.34
pOX38A::CAT + pLF107	7.90	8.30×10^{-3}	951.81
F*lac traL311* + pLF164	53.45	4.34×10^{-3}	1231.22
F*lac traL311* + pLF107	60.77	6.19×10^{-3}	982.25
F*lac traE18* + pRS27	3.13	4.81×10^{-3}	650.73
F*lac traE18* + pKAR-3	0.13	1.43×10^{-4}	792.68
F*lac traK105* + pRS27	0.002	5.77×10^{-6}	346.67
F*lac traK105* + pKAR-1	0.25	6.05×10^{-4}	413.04
F*lac traB2* + pRS27	3.75	6.64×10^{-3}	565.11
F*lac traB2* + pKAR-1	2.34	3.22×10^{-3}	725.72
F*lac traV569* + pKI164	24.1	2.70×10^{-2}	873.63
F*lac traV569* + pKAR-1	1.46	2.08×10^{-3}	702.96
F*lac traC5* + pRS29	0.67	1.47×10^{-3}	457.04
F*lac traC5* + pKAR-1	13.06	1.70×10^{-2}	757.84
F*lac traW546* + pRS29	37.33	1.57×10^{-2}	2372.81
F*lac traW546* + pKAR-1	15.24	8.66×10^{-3}	1760.02

Table 5.2 Cont.

pOX38-*traU347*+ pRS29	14.03	2.38×10^{-2}	587.35
pOX38-*traU347*+ pKAR-1	24.25	4.64×10^{-2}	522.63
pOX38-*trbC460* + pRS29	0.56	3.25×10^{-4}	1722.45
pOX38-*trbC460* + pKAR-1	18.43	8.74×10^{-3}	2108.25
F*lac traF13* + pKI175	100.00	1.03×10^{-1}	967.74
F*lac traF13* + pKAR-4	100.00	6.65×10^{-2}	1503.76
pOX38-*traQ238* + pRS29	1.18	1.42×10^{-3}	830.99
pOX38-*traQ238* + pKAR-5	8.86	7.51×10^{-3}	1179.76
F*lac traH80* + pRS29	0.29	2.00×10^{-4}	1450.00
F*lac traH80* + pKAR-5	22.70	1.91×10^{-2}	1188.48
F*lac traG106* + pRS1670	27.55	4.76×10^{-3}	5787.81
F*lac traG106*+ pKAR-6	10.00	1.07	9.36
pOX38-*traX482* + pKAF-3	100.00	1.97×10^{-2}	5076.14
pOX38-*traX482* + pKAR-7	100.00	1.80×10^{-2}	5555.56

[a] The mating efficiencies of F *tra* mutants to recipients harbouring either pSC101 vector control or to recipients expressing TraT and TraS from pRS31, are indicated.

[b] Surface Exclusion index was calculated by dividing the mating efficiency to recipients carrying pSC101 with the mating efficiency to recipients carrying pRS31.

[c] The reported numbers represent the mean values obtained in at least three experiments where the sample values did not exceed 30% of the mean.

however, recognizes only the acetylated F pilin since its epitope comprises the acetylated octapeptide that corresponds to the N-terminal sequence of mature pilin [(Section 1.4.4, Fig. 1.5; Frost *et al.*, 1986)]. In the Western blots shown in Fig. 5.2, JEL92-reactive pilin is present in all except the negative control, MC4100 cells (Panel B, lane 5). However, JEL93-reactive pilin is absent in cells harbouring pOX38-*traX482* (Panel A, lane 2) but present when either pKAR-7 or pKAF-3 plasmids are supplied in trans (Panel A, lanes 3 and 4 respectively). These results indicate that R100-1 *traX* can complement pOX38-*traX482* mutation and effectively acetylate F pilin subunits. As shown in Tables 5.1 and 5.2, cells harbouring pOX38-*traX482* and pKAR-7 retained normal sensitivity to pilus-specific phages and exhibited an F-like surface exclusion specificity. Analysis by TEM revealed that pOX38-*traX482* pili attached both f1 and R17 phages in aggreement with the results of Maneewannakul *et al.* (1995; data not shown).

5.2.5 Immunological characteristics of F pili containing unacetylated pilin subunits

To ascertain if the pili produced by pOX38-*traX482* which are composed of unacetylated pilin subunits, are structurally dissimilar than the pili of pOX38-Km, the ability of mAb JEL92 to laterally decorate these pili was tested by immunogold electron microscopy (Section 4.4). *E. coli* MC4100 cells harbouring either pOX38-*traX482* or pOX38-Km plasmids were incubated with mAb JEL92, then following incubation with anti-mouse goat IgG conjugated to colloidal gold, they were examined by TEM. Fig. 5.3 depicts electron micrographs of pili of pOX38-Km (A) and pOX38-*traX482* (B) labelled with mAb JEL92. As previously shown (Frost *et al.*, 1986), mAb JEL92 selectively binds to the basal knobs but not to the sides of pOX38-Km pili (A). Similarly, only the vesicle-like structures but not the filaments of pOX38-*traX482* pili attach mAb JEL92. Based on these results, both pOX38-Km and pOX38-*traX482* elaborate structurally similar pili which do not attach JEL92 laterally.

Fig. 5.2 Western blot analysis of F pilin acetylation. Total cellular proteins from *E. coli* MC4100 cells harbouring pOX38-Km (lane 1), pOX38-*traX482* (lane 2), pOX38-*traX482* + pKAF-3 (lane 4), pOX38-*traX482* + pKAR-7 (lane 4) or MC4100 (lane 5) were separated on 15% SDS-polyacrylamide gels. Western blot analysis was performed as described in Section 4.4. F pilin was detected with either mouse mAb JEL93 (Panel A) or mAb JEL92 (Panel B) and anti-mouse secondary antibody HRP-conjugate. Molecular mass markers in kDa are shown on the left.

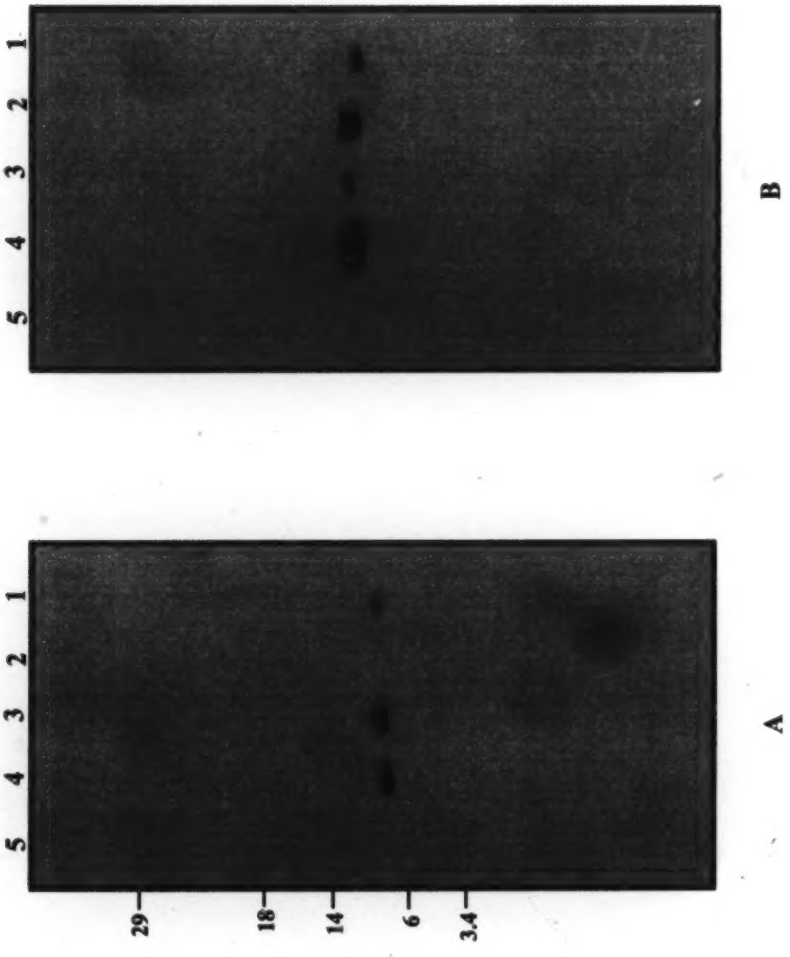
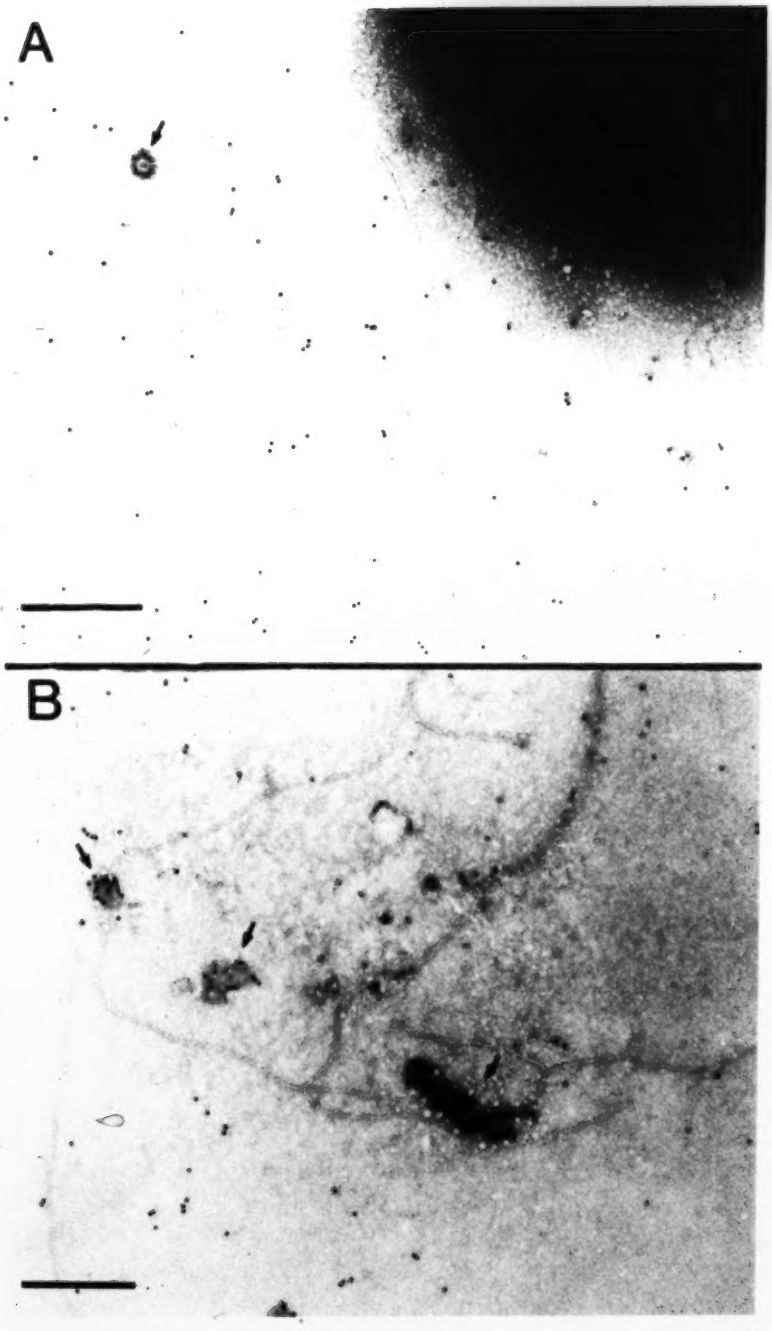

5 4 3 2 1
B
5 4 3 2 1
29
18
14
6
3.4
A

Fig. 5.3 Serological characteristics of F pili composed of either acetylated or unacetylated pilin subunits. Immunogold electron microscopy was carried out as described in Section 5.4. The attachment of mAb JEL92 to pili elaborated by MC4100 cells harbouring either pOX38-Km (A) or pOX38-*traX482* (B) was detected with anti-mouse secondary antibody conjugated to colloidal gold. Arrows indicate terminal knobs or vesicular materials coated with gold particles. Total magnification is 14 500x and the size bar represents 1 μm.


A
B

5.3 DISCUSSION

In contrast to the gene products involved in conjugal DNA metabolism which define plasmid specificity, the proteins for pilus synthesis are interchangeable between F and R100-1 plasmids. Previously, I have demonstrated that the allelic differences in the pilin gene, *traA*, do not specify the functional differences between F and R100-1 pili (Chapter 2). To determine if the allelic variation among the remaining pilus synthesis genes defines the functional specificity of their pili, trans-complementation studies with F *tra* mutants carrying specific mutations in each of the pilus synthesis genes were carried out. Chimeric plasmids expressing either F or R100-1 pilus synthesis genes were used to complement each of the mutations and the effect of the R100-1 allele on sensitivity to pilus-specific phages and surface exclusion specificity was measured.

The results revealed that none of the pilus synthesis genes tested gave rise to a phenotype whose phage sensitivity profile matched that of R100-1, suggesting that the differences in the primary sequence of the pilus synthesis genes do not determine susceptibility to f1 and R17 infection. As such, a genetic basis for the phenotypic variation between F and R100-1 pili may not exist. Thus, it seems possible that the pilin subunits in F and R100-1 pili might be assembled in a slightly different conformation and that this conformational difference governs the adsorption and subsequent infection by these phages following pilus retraction. The strongest support for this notion comes from the observation that pOX38A::CAT expressing R100-1 pilin retained full susceptibility to both f1 and R17 infection (Chapter 2). The current data is also in full agreement with the findings of the previous chapter that a unique pilus tip protein thought to be the receptor for f1 phage does not exist in F pili.

The surface exclusion specificity of F-like plasmids has been directly correlated with the respective pilus-type such that exclusion usually occurs when the pilus-type of the incoming plasmid matches the surface exclusion specificity of the co-resident plasmid (Section 1.5.3). Analyses of DNA transfer by F *tra* mutants expressing R100-1 pilus synthesis genes to recipient cells expressing both F TraT and TraS proteins revealed that the surface exclusion specificity of F and R100-1 plasmids is determined exclusively by the TraG

protein. The F *traG* product is unique among the transfer proteins since it is required for both pilus assembly and mating aggregate stabilization. Mutations that affect the N-terminus of the protein prevent pilus assembly whereas mutations affecting the C-terminus, although they do not alter the synthesis of normal pili, severely impair the formation of stable mating aggregates and thus drastically reduce DNA transfer (Manning *et al.*, 1981). The *traG* product is approximately 100 kDa and is comprised of two large periplasmically-located regions which are anchored to the inner membrane by three predicted transmembrane segments (Firth and Skurray, 1992). The pilus assembly function is carried out only by the N-terminal 60% of the protein which presumably acts as an integral element of the transenvelope pilus assembly complex (Section 1.7). A 50 kDa product arising from internal cleavage of the full length TraG has been identified. The so-called TraG* corresponds to the C-terminal sequences of TraG which are required for the mating aggregate stabilization function of the protein. Consistent with its periplasmic location, TraG* has been postulated to act in concert with the outer membrane protein, TraN, the only other *tra* protein demonstrated to be involved in aggregate stabilization (Firth and Skurray, 1992).

As previously described in Section 1.5.3, surface exclusion specificity is determined by the independent actions of TraT and TraS; the former reduces stable mating aggregate formation while the latter prevents donor conjugal DNA transmission in a plasmid-specific manner. Approximately 10-20 fold reduction in DNA transfer is observed when TraT alone is present in the recipient cell while the action of TraS alone can reduce DNA transfer by 100-200 fold (Achtman *et al.*, 1980). Although the cumulative effects of both TraT and TraS were measured in the surface exclusion assays described in this study, the Sfx index of F*lac traG106* complemented with R100-1 *traG* is similar to that of the R100-1 plasmid itself (Table 5.4), suggesting that perhaps both TraT- and TraS-mediated surface exclusion acts in a TraG-specific manner. It is conceivable that TraG* might interact directly with TraT in the outer membrane of recipient cells and this interaction, depending on the plasmid exclusion specificity, might determine if stable mating aggregates will be formed. Concurrently, TraG* or TraG might directly interact with TraS in the inner membrane of recipient cells in a sequence-specific manner and this interaction may in turn block the transmission of the

mating signal and prevent conjugal DNA metabolism. Although it is evident from this study that TraG plays a role in surface exclusion, rigorous biochemical analysis of the protein is required to elucidate the exact mechanism of its function. For instance, segment swapping experiments to create a hybrid TraG protein with the N-terminal sequence of the F plasmid and the C-terminal sequence from R100-1 plasmid would be worthwhile in discerning which domain of the protein is important for surface exclusion. Further, the transfer of F*lac traG106* complemented with R100-1 *traG* to recipient cells expressing either TraT or TraS proteins alone should be measured in order to ascertain if the surface exclusion specificity is solely determined by TraG.

Contradictory to earlier suggestions that pili composed of unacetylated pilin subunits are structural variants of wild-type pili, the pOX38-*traX482* plasmid synthesized pili that were identical to wild type pili in their phage adsorbing characteristics and in their inability to bind mAb JEL92 laterally. Immunogold electron microscopy revealed that the mAb JEL92 did not decorate the sides of pOX38-*traX482* pili but bound specifically to the vesicle-like structures composed of unassembled pilin often found at the base of F pili. These results confirmed that both wild-type and pOX38-*traX482* pili are structurally identical and that acetylation of F pilin does not alter its conformation in the pilus filament.

In addition to an increased tendency to form lateral aggregates and poor capacity to adsorb R17 phages, Grossman *et al.* (1990) demonstrated that the pili synthesized by the pTG801 plasmid attached both mAb JEL92 and JEL93 to their sides. Contradictory to the latter finding however, Moore *et al.* (1993) demonstrated that pilin synthesized by pTG801 plasmid do not react with mAb JEL93 in agreement with my results and that of Maneewannakul *et al.* (1995) that unacetylated F pilin lacks this epitope. Thus, interaction between mAb JEL93 and pTG801 pili may represent a background reaction or residual affinity of mAb JEL93 for N-terminal pilin amino acid residues that could be detected only by electron microscopy (Moore *et al.*, 1993). My results also show that the peculiar characteristics of pTG801 pili are not due to the absence of pilin N^{α}-acetylation since pili synthesized by pOX38-*traX482* are structurally similar to wild-type pili. Since mutations affecting the genes distal to *traG* do not alter the function or the physical characteristics of

pili, the absence of *traS*, *traT*, *traD*, *trbH*, *traI*, *traX* and *finO* in pTG801, theoretically should not affect the pilus structure. However, the observation that pRS31 which supplies the missing *tra* region, when co-resident with pTG801 restored wild-type characteristics to pTG801 pili, strongly suggests that additional unidentified elements that are important for determining pilus number and correct pilus assembly are present in pRS31. Although copy number and promoter differences cannot be discounted, the pOX38-*tra715* plasmid in which the entire *tra* operon is expressed by the strong bacteriophage T7 promoter (Section 4.1), synthesized, on average, 1 pilus per cell despite substantial increase in *tra* mRNA levels and protein expression (Maneewannakul *et al.*, 1992). Similar results were also obtained by Johnson and Willetts (1980) upon cloning the whole *tra* region into the multicopy number vector, pBR322. Consistent with this proposal, inner membrane preparations of pTG801 hosts contained less pilin when compared to F' hosts where 85% of pilin was localized in the inner membrane (Moore *et. al.*, 1993; Section 1.4.3) indicating increased pilin utilization. Thus, the anomaly in pTG801 pili could be attributed to an overall perturbation in the packing arrangements of pilin subunits in the filament due to increased pilin utilization resulting in structurally variant pili.

The notion that N^{α}- acetylation of pilin is plasmid-specific and contributes to pilus variation was negated since R100-1 *traX* acetylated F pilin and the pili synthesized retained F-like characteristics. Although a definitive role for pilin acetylation is not obvious in the assembly of conjugative pili, the general function of N^{α}- acetylation in eukaryotic proteins is to afford protection from proteolytic degradation (Jornvall, 1975). It is also conceivable that the acetyl group might play a role in 'registration' or recognition during assembly by interacting with the next subunit in the fibre. Analysis of cytoplasmic actin in *Drosophila* cells indicated that either acetylated or unacetylated actin can participate in the assembly of microfilaments but the latter is used less efficiently (Berger *et al.*, 1981). Even though acetylation is not critical for F pilus assembly, it will be interesting to determine if the non-acetylated pilin subunits are assembled independently of their acetylated counterparts or if both can be co-polymerized to form a heterogeneous filament. Possibly, pilin acetylation is

more critical to assembly, stability or function of pili in other hosts or under other physiological conditions (Maneewannakul *et al.*, 1995).

5.4 MATERIALS AND METHODS

5.4.1 Bacterial strains and plasmids

The *E. coli* strains used in this study include ED2149 [F *tsx lacΔU124 Δ (nadA aroG gal att bio),* Achtman *et al.*, 1972], ED24 (F Lac Spc^R, Achtman *et al.*, 1971), XK1200 (Table 2.4) , JE-1 (Table 2.4), MC4100 (Table 2.4) and DH5α (Section 4.4). A list of F plasmid derivatives carrying mutations in *tra* and *trb* genes involved in pilus synthesis and a description of all the plasmids used in this work are given in Tables 5.3 and 5.4, respectively.

5.4.2 Bacterial manipulations, recombinant DNA techniques and PCR

Techniques for bacterial transformation and PCR are as described in Sections 2.4 and 3.4. Restriction endonuclease digestion, conversion of recessed 3' termini or staggered DNA termini to generate blunt ends and ligation of DNA molecules were performed as described by Ausubel *et al.*, 1989.

5.4.3 Immunogold electron microscopy

The procedure for immunogold electron microscopy is as described in Section 4.4. The mAb JEL92 was used at 1:500 dilution and gold-conjugated anti-mouse goat antibodies were used at 1:100 dilution.

5.4.4 Qualitative phage tests, mating and surface exclusion assays

f1 and R17 phage sensitivity assays and mating experiments were performed as described in Section 2.4. Surface exclusion was determined from mating experiments with F*lac*, *kan* and CAT insertion mutants (Table 5.3) complemented with the appropriate plasmids (Table 5.4) as donors, and ED24 recipients carrying either the vector, pSC101, or the plasmid pRS31 which expresses both TraS and TraT surface exclusion proteins. F*lac* donors were selected on MacConkey medium (Sambrook *et al.*, 1989) containing either ampicillin (50 μg/ml) or

Table 5.3 Derivatives of the F plasmid carrying mutations in *tra* and *trb* genes involved in pilus synthesis

Gene	F*lac* mutant[a]	*kan*[b] or *CAT*[c] insertion mutant	Reference
traA		pOX38*A::CAT*	Anthony *et al.*, 1994
traL	F*lac traL311*[d]		Willetts, 1973
traE	F*lac traE18*		Willetts and Achtman, 1972
traK	F*lac traK105*[d]		Willetts and Achtman, 1972
traB	F*lac traB2*		Willetts and Achtman, 1972
traV	F*lac traV569*		Doran *et al.*, 1994
traC	F*lac traC5*		Willetts and Achtman, 1972
traW	F*lac traW546*		Miki *et al.*, 1978
traU		pOX38-*traU347*[b]	Moore *et al.*, 1990
trbC		pOX38-*trbC460*[b]	Maneewannakul *et al.*, 1991
traF	F*lac traF13*		Willetts and Achtman, 1972
traQ		pOX38-*traQ238*[b]	Kathir and Ippen-Ihler, 1991
traH	F*lac traH80*		Willetts, 1973
traG	F*lac traG106*[d]		Firth and Skurray, 1992
traX		pOX38-*traX482*[b]	Maneewannakul *et al.*, 1995

[a]Except where noted, all F*lac tra* mutants carry an amber mutation. These mutants were tested in strain ED2149.

[b] All *kan* (kanamycin resistance gene cassette) and the [c]*CAT* (chloramphenicol acetlytransferase gene cassette) insertion mutants were tested in strain AK1200.

[d] *traK105* and *traG106* are frameshift mutations and *traL311* is a UGA nonsense mutation (Moore *et al*, 1993).

Table 5.4 Plasmids used in this study

Plasmid	Description	Reference/Source
pKAR-1[a]	11.569 kb *Eco*RI fragment from R100-1 (containing *traK-trbC*) in pT7.4; Ap^R	This work
pKAR-2	2.987 kb fragment from R100-1 (carrying *traK-traP* genes) cloned in *Bam*HI-*Eco*R1 sites of pUC118; Ap^R	Table 3.4
pKAR-3[a]	2.4 kb *Bam*HI-*Pst*I fragment from R100-1 (carrying *traY-traK* genes) in pACYC177; Km^R	This work
pKAR-4[a]	2.1 kb *Sma*I-*Sac*I fragment from R100-1 (carrying *trbE-trbA* genes) cloned in *Sma*I site of pACYC177; Ap^R	This work
pKAR-5[a]	5.1 kb *Sac*I-*Eco*RI fragment from R100-1 (carrying *traQ-traH* genes) cloned in *Sma*I-*Hin*dIII sites of pACYC177; Ap^R	This work
pKAR-6	2.989 kb fragment from R100-1 carrying *traG* cloned in *Bam*HI-*Hin*dIII sites of pT7.3; Ap^R	This work
pKAR-7	0.817 kb fragment from R100-1 carrying *traX* cloned in *Pst*I-*Hin*dIII sites of pT7-3; Ap^R	This work
pKAF-2	3 kb fragment containing *traK-traP* from F cloned in *Bam*HI-*Eco*RI sites of pUC118; Ap^R	Table 3.4
pKAF-3	0.884 kb fragment carrying F *traX* cloned in *Sma*I-*Hin*dIII sites of pT7-3; Ap^R	This work
pLF164	F *traALE* in pUC8, Ap^R	Table 2.4
pLF107	R100-1 *traALE*::IS2 in pUC8, Ap^R	Table 2.4
pKI164	2.55 kb *Hinc*II fragment carrying F *traP-traR* genes in pACYC177; Ap^R	Moore *et al.*, 1987
pKI175	6.2 *Ava*I fragment carrying F *traW-traQ* genes in pACYC177; Ap^R	Wu *et al.*, 1987
pRS1670	F *traG* in pGEX-1 vector; Ap^R	Firth and Skurray, 1992
pRS29	*Eco*RI f1 and f15 fragments from F in pSC101; Tc^R (carries *traV-traH* genes)	Skurray *et al.*, 1976

Table 5.4 cont.

pRS27	*Eco*RI f6 and f15 fragments from F in pSC101; Tc^R (carries *traM-traR* genes)	Skurray *et al.*, 1976
pRS31	*Eco*RI f2, f17 and f19 fragments from F in pSC101; carries *traS-finO::IS3* genes, Tc^R	Skurray *et al.*, 1976
pBluescript SK+	ColE1 ori; Ap^R	Stratagene
pACYC-177	P15A ori; Ap^R, Km^R	Chang and Cohen, 1978
pT7-3 & pT7-4	ColE1 ori; Ap^R	Tabor and Richardson, 1985
pSC101	Tc^R	Cohen and Chang, 1977
pOX38-Km	pOX38-Km Tra^+ RepFIA$^+$ Km^R f1*Hin*dIII fragment of F plus *Hin*dIII fragment of Tn*5*	Table 2.4
R100-1	R100-1 Tra^+ Cm^R Fa^R Sm^R Sp^R Su^R Tc^R	Table 2.4

[a] The sizes of the inserts in pKAR-1, pKAR-3, pKAR-4 and pKAR-5 were calculated from the locations of restriction endonuclease sites reported in Womble and Rownd, 1988.

kanamycin (25 µg/ml) while transconjugants were selected on M9 minimal medium (2% agar, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 3 g $CaCl_2$ and 1 mM $MgSO_4$ per 1 l H_2O; Ausubel *et al.*, 1989) supplemented with 0.6% lactose and 20 µg/ml tetracycline. In matings with *kan* insertion mutants, donors were selected on LB agar containing both ampicillin and kanamycin while transconjugants were selected on LB agar containing kanamycin and tetracycline. Donors carrying the pOX38A::CAT plasmid (Section 2.4) were selected on LB medium containing 25 µg/ml chloramphenicol and ampicillin while the transconjugants were selected on LB agar containing chloramphenicol and tetracycline. Surface exclusion indices were calculated as the mating efficiency obtained with recipient cells carrying pSC101 divided by the mating efficiency obtained with recipient cells carrying pRS31 as previously described in Section 2.4. Control experiments with donors carrying either pOX38-Km or R100-1 plasmids were also carried out simultaneously. Donors were selected on LB agar containing kanamycin and nalidixic acid (40 µg/ml) and transconjugants on LB agar containing kanamycin and tetracycline for matings with pOX38-Km plasmid. To determine surface exclusion against the R100-1 plasmid, ED24 cells carrying either pSC101 or pRS31 and the vector pT7-3 were used as recipients. The pT7-3 vector provided an ampicillin marker for the selection of transconjugants. In these matings, the donors were selected on LB agar containing streptomycin (20 µg/ml) and nalidixic acid while LB agar containing streptomycin, tetracycline and ampicillin (30 µg/ml) was used for the selection of transconjugants.

5.4.5 Construction of pKAR series of chimeras carrying pilus synthesis genes from R100-1 plasmid

The R100-1 plasmid was used as the source of DNA to generate the pKAR series of chimeras. pKAR-1 was constructed by digesting approximately 20 µg of plasmid DNA with *Eco*RI and the resulting fragments were ligated into the *Eco*RI site of pT7-4 vector (Table 5.4). Colonies carrying a 11.6 kb fragment spanning the *traK-trbC* region were identified by colony hybridization with [γ^{32}P]-end-labelled oligonucleotide primer, LFR39 (Section 3.4) as described in subsequent sections. The presence of the insert was confirmed by restriction

mapping. The resulting plasmid, pKAR-1 contained the insert in the same orientation as the ϕ6 promoter on the vector. The 11.6 kb fragment was further digested with *Eco*RI and *Sal*I enzymes to generate two fragments approximately 7.6 kb and 4 kb in size. These were subsequently cloned into pBluescript SK+ vector (Table 5.4) and their nucleotide sequences were determined by DNA sequencing (Section 3.4). pKAR-3 was constructed in a similar manner by shotgun cloning *Bam*HI and *Pst*I digested R100-1 plasmid DNA into pACYC177 vector (Table 5.4) digested with the same enzymes. Colonies carrying a 2.4 kb fragment spanning the *traY-traE* region were identified by colony hybridization using [γ^{32}P]-end-labelled KAN-5 oligonucleotide primer (5'-GCGACCTTCGGTAAGGA-3'). The insert was sub-cloned into pBluescript KS+ vector to determine its nucleotide sequence. pKAR-4 was created by sub-cloning a 2.03 kb *Sma*I-*Sac*I fragment into the *Sma*I site of pACYC177. This fragment was derived from the parent plasmid pBK-7 (Klimke and Frost, unpublished results) which contains a 6 kb *Sal*I-*Sac*I fragment spanning the *trbI-trbA* region. The DNA sequence of the 2.03 kb insert was determined following sub-cloning of the fragment into pBluescript SK+. A 5.1 kb *Sac*I-*Eco*RI fragment containing *traQ-traH* genes was initially cloned into pT7-3 vector (Table 5.4) as described previously for the construction of pKAR-1. This insert was excised by digestion with *Sac*I and *Hin*dIII enzymes and was subsequently cloned into *Sma*I-*Hin*dIII sites of pACYC177 to give rise to pKAR-5. The *Sac*I-*Hin*dIII fragment was also cloned into pBluescript SK+ for DNA sequence determination. The construct pKAR-6 was created by amplifying a 3 kb fragment containing the *traG* gene by PCR using the oligonucleotide primers KAN-G1 (5'-CGCGGATCCGCATTGCTGGTTGTTGACCGTC-3') and KAN-G2 (5'-GCCCAAGCTTGTATCACCGTTACCAGAGTAATATG-3') and R100-1 plasmid DNA as the template. Using the *Bam*HI and *Hin*dIII sites provided in the primers, this fragment was subsequently cloned into pT7-3. The DNA sequence of *traG* was also determined following sub-cloning of the fragment into pBluescript KS+. Similarly, the *traX* gene was amplified by PCR using the oligonucleotide primers KAN-X1 (5'-CGCGCCCGGGAAAATCTGCTGCAGGAGCG-3') and KAN-X3 (5'-GCCCAAGCTTCAGAATCAGCGTCAGTATCATACCAG-3') whose sequences were derived from the nucleotide sequence of R100-1 *traX* reported by Yoshioka *et al.* (1990). This

fragment was restricted with *Pst*I and *Hin*dIII enzymes and was subsequently cloned into pT7-3 to create pKAR-7. The DNA sequence of *traX* was confirmed following sub-cloning of this fragment into pBluescript KS+.

5.4.6 Construction of pKAF-3

In order to create a chimeric plasmid carrying the F *traX* gene, a 884 bp fragment was amplified by PCR using primers KAN-X1 and KAN-X2 (5'-GCCCAAGCTTGCGGCCAAAGATACCACACCCGTCAC-3') and the plasmid pRS31 as the template DNA. This fragment was cloned into the *Sma*I-*Hin*dIII sites of pT7-3 to create pKAF-3. Upon re-cloning into pBluescript KS+ the DNA sequence of *traX* was determined.

5.4.7 Identification of recombinant clones by colony hybridization

Following ligation of DNA molecules, the ligation mixture was electroporated into competent DH5α cells as described in Section 2.4. Positive clones were identified on colony blots performed using the method of Grunstein and Hogness (1975). Nylon membrane disks (Hybond™-N, Amersham Life Science) were placed onto agar plates with well spread transformant colonies, marked for orientation and left until evenly wet. After removal of the membrane disks, the plates were incubated at 37°C to allow colony regrowth. The membranes were incubated colony-side up for 5 min sequentially on sheets of 3 MM Whatman paper that had been soaked in 10% SDS (w/v); 0.5 M NaOH/1.5 M NaCl; 1.5 M NaCl/0.5 M Tris-HCl pH 8.0; and finally SSPE buffer (8.8 g NaCl, 1.4 g $NaH_2PO_4.H_2O$, 0.37 g EDTA per 1l, pH 7.4; Sambrook *et al.*, 1989). The membranes were air-dried then the DNA was cross-linked under UV in a Gene Pulser (150 mJ, Bio-Rad laboratories). The membranes were pre-hybridized in Hybridization solution [5x SSC (Section 2.4), 1 mM EDTA, 0.1% SDS and 1x Denhardt's solution (the composition of 50x Denhardt's solution is 1% BSA (w/v), 1% ficoll (w/v), 1% polyvinylpyrrolidone (w/v), Sambrook *et al.*, 1989)] containing 0.1 mg/ml heat denatured calf thymus DNA at 37°C for a minimum of 1 hr. Following pre-hybridization, approximately $1x10^6$ cpm of radioactively labelled probe was added and incubation was continued overnight. The membranes were washed once in 5xSSC

containing 0.1% SDS at 37°C for 15 min followed by successive washings at temperatures 5°C higher until the background radioactivity was reduced. The membranes were air-dried prior to autoradiographic development. Positive signals were correlated to colonies on the agar plates and the plasmid DNA from these colonies was isolated (Sambrook *et al.*, 1989) and analyzed by restriction endonuclease digestion and DNA sequencing.

5.4.8 Preparation of radioactively end-labelled oligonucleotide probes

Approximately 100 pmol of the oligonucleotide primer to be labelled, 1 µl 10x PNK buffer (Boehringer Mannheim) and 100 µCi of [γ^{32}P]-ATP (7 000 Ci/mmol; Mandel Scientific, NEN™ Life Science Products) were mixed in a total volume of 10 µl. One unit of T4 polynucleotide kinase (PNK, Boehringer Mannheim) was added and the mixture was incubated at 37°C for 30 min. Then one more unit of PNK was added and incubation was continued for a further 30 min. The volume of the labelling reaction was brought to 70 µl with the addition of STE buffer (100 mM NaCl, 20 mM Tris-HCl, 10 mM EDTA, pH 7.5) and the probe was purified on a Nuc-Trap push column (Stratagene) and counted in a scintillation counter to determine the efficiency of labelling.

5.4.9 Large scale purification of R100-1 plasmid DNA

The 60 kb R100-1 plasmid was purified using the Triton-Lysis method. A 2 ml overnight culture of *E. coli* JE-1 harbouring the R100-1 plasmid was inoculated into 1 l of LB broth containing streptomycin (20 µg/ml) and the culture was grown overnight. Cells were harvested by centrifugation at 2 000 g for 15 min at 4°C. The cell pellet was resuspended in 20 ml of 25% sucrose, 50 mM Tris-HCl pH 8.0 and incubated on ice for 5 min. Three ml of lysozyme solution (20 mg/ml lysozyme in 0.25 M EDTA pH 8.0) was added, mixed by inversion and the mixture was further incubated on ice for 5 min. Then 20 ml of 0.25 M EDTA pH 8.0 were added and again mixed by inversion and incubated on ice for 30 min. Following the addition of 30 ml of Triton-lysis solution (4 ml Triton X-100, 50 ml 0.25 EDTA pH 8.0, 10 ml 1 M Tris-HCl, pH 8.0, 2 ml 5 mg/ml RNaseA /per 200 ml solution), the mixture was further incubated on ice for 60 min with periodic mixing by gentle inversion.

The supernatant was collected following centrifugation at 12 100 g for 2 hrs at 4°C. After the addition of 12 ml of 50% PEG-8000 and 4 ml of 5 M NaCl, the DNA was precipitated at 4°C overnight. The precipitate was collected following centrifugation at 3 030 g for 15 min at 4°C. The precipitate was dissolved in 23 ml of CsCl in TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH 7.5; density 1.397 g/cm^3) and was recentrifuged at 12 100 g for 15 min at 4°C. The supernatant was then transferred to a solid-wall ultracentrifuge tube to which 200 µl of ethidium bromide (10 mg/ml) was added. The gradient was set up in a 50.2 Ti fixed angle rotor and was run in a Beckman ultracentrifuge at 38 000 rpm for 48 hrs at 18°C. The lowest bands were extracted and were dialyzed extensively in TES buffer (40 ml 1 M Tris-HCl pH 8.0, 16 ml 0.25 M EDTA pH 8.0, 8 ml 0.5 M NaCl/ per 1 l H_2O). Following phenol extraction, the DNA was recovered by ethanol precipitation and stored in TE buffer at -20°C.

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CHAPTER 6

General Discussion

6.1 DISCUSSION

The broad aim of the studies outlined in this thesis was to add to our fundamental knowledge of the conjugal process encoded by the F plasmid. The experiments described herein were performed specifically to gain insights into the role of the pilus in conjugation. To that end, a combination of genetic analysis of the gene products involved in pilus synthesis, and direct physical characterization of the F pilus itself were undertaken to elucidate a genetic basis for the phenotypic variation between the pili of F and R100-1 plasmids in pilus-mediated processes, specifically in recipient cell recognition, surface exclusion and pilus-specific bacteriophage infection. The analyses performed have yielded the following results. The findings of Chapter 2 revealed that contrary to the widely accepted notion, the sequence differences at the N-terminus of F-like pilin do not define the plasmid-specificity in recognition of either LPS receptors or OmpA on recipient cells or the surface exclusion protein, TraT or determine the degree of susceptibility to both DNA and RNA phages. This unexpected finding was the impetus to ensuing experiments aimed at identifying one or more pilus synthesis genes whose allelic differences might form the genetic basis for the phenotypic variation of F and R100-1 pili. In Chapter 3, the nucleotide sequence of the pilus assembly genes *traK, B* and *P* from R100-1 was determined. Amino acid sequence comparisons of TraL, E, K, B and P proteins of F, ColB2 and R100-1 plasmids revealed sequence variations among these proteins which might define pilus specificity. This work also provided the starting point for further determination of the nucleotide sequence of the *tra* region of R100-1 plasmid in order to catalogue the allelic differences among the pilus synthesis genes of F and R100-1 plasmids. Chapter 4 describes the results obtained from an affinity purification procedure designed to isolate the M13 phage receptor at the F pilus tip. Lack of a unique tip adhesin in F pilus suggested alternate possibilities for the nature of the phage receptor, hence the pilus tip. Finally, the results obtained from a detailed complementation analysis of F plasmid derivatives carrying lesions in each of the fifteen pilus synthesis genes with the corresponding allele from the R100-1 plasmid are presented in Chapter 5. In addition to the apparent lack of functional specificity among the pilus

synthesis genes, these results revealed that the surface exclusion specificity between F and R100-1 plasmids is determined by the bifunctional protein, TraG.

The information gathered from this study as well as further work carried out on F pilin and the TraN protein have shed more light into the nature of the pilus and its role in conjugation. The remainder of this chapter will therefore focus on these aspects from a broad perspective. Further, the possibility that the F pilus and associated proteins play a role in the conjugation process beyond recognition of the recipient cell is discussed in light of the evolutionary relationships that have recently become apparent between F plasmid conjugation and a number of bacterial transport systems.

6.1.2 The role of the pilus in bacteriophage infection and the nature of the pilus tip

Despite the notable differences in the plating efficiencies of f1 and R17 phages by cells harbouring either F or R100-1 plasmids (Chapter 2), the complementation experiments described in Chapters 2 and 5 failed to identify a pilus synthesis protein that determines the variation in phage sensitivity. In addition, the pili synthesized by pOX38A::CAT expressing R100-1 pilin conferred full sensitivity to both phages even though R100-1 pili inherently do not attach R17 phage (Chapter 2). Taken together, these results suggest that the phenotypic variation in phage susceptibility conferred by F and R100-1 pili, is not due to sequence-specific alterations in a single pilus synthesis gene but rather due to intrinsic differences in either the architecture or the assembly/retraction kinetics of their pili brought about by a complement of genes.

The absence of an F pilus tip adhesin as the receptor for f1 phage suggests the possibility of a unique tip-specific arrangement of pilin subunits where specific residues in pilin that are exposed at the tip mediate interactions with the phage pIII protein. Indirect support for this contention comes from the studies of Malmborg *et al.* (1997) where the insertion of a 13 aa peptide coding for the AD-2 epitope of a cytomegalovirus glycoprotein at the C-terminus of F pilin, resulted in the synthesis of pili that were unable to attach wild-type M13 phage. However, an M13 derivative displaying an AD-2-specific antibody fragment on its pIII successfully infected cells expressing the recombinant pili. Although the

authors did not demonstrate whether the AD-2 epitope is exposed at the tip or the sides of pili, the C-terminal extension in F pilin appears to have altered the normal pIII attachment site, thus preventing infection by wild type phage. These results implicate a direct role for pilin in M13 phage attachment. Recently, in a collaborative work with this laboratory, Rondot and Breitling (University of Heidelberg, Germany) inserted random or specific amino acid sequences ranging from 1 to 56 aa at the C-terminus of F pilin and tested for the production of functional recombinant pili. The results revealed that several insertions abolished pilus assembly altogether, while several more although they prevented pilus assembly, conferred M13 phage sensitivity suggesting the presence of a tip structure at the cell surface. Other insertions resulted in the production of a reduced number of pili which did not display the foreign peptide on the surface, while a specific 17 aa insertion gave rise to pili that were proficient in conjugation and M13 phage adsorption, and displayed the peptide laterally (Rondot *et al.*, 1998). Although the specific requirements for efficient display of foreign peptides on the surface of F pili could not be discerned from this study, it nevertheless highlights the possibility of inserting specific epitopes at various sites in mature F pilin in order to identify the domain that is exposed at the tip. The region spanning the highly conserved hexapeptide $V_{16}KATFG_{21}$ among F-like pilin (Section 1.4.5) could be targeted as a preliminary site of insertion and if the epitope is exposed at the tip, site-directed mutagenesis of this region could be carried out to delineate the residues which are important for M13 phage attachment.

6.1.3 Role of the pilus in recipient cell recognition

Since an extracellular pilus is a prerequisite for the formation of a mating pair, it was believed that the pili of F-like plasmids recognize distinct substituents on the LPS or OmpA of recipient cells. However, it is increasingly becoming apparent that the pilus tip may merely initiate non-specific contacts with the recipient cell envelope to bring the mating cells into wall-to-wall proximity following pilus retraction. Recent findings in our laboratory (Klimke and Frost, 1998) revealed that both LPS and OmpA which were previously believed to be the receptors for the F pilus (Section 1.5.2), are important components of the recipient cell

required for stabilization of mating aggregates in liquid medium after wall-to-wall contact has been established. By complementing an F plasmid derivative carrying a null mutation in the mating aggregate stabilization gene, *traN*, with the corresponding allele from R100-1 plasmid, Klimke and Frost (1998) demonstrated that unlike the F plasmid, this construct transferred at high levels to recipient cells carrying mutations in either *rfaP* or *ompA* loci, analogous to the R100-1 plasmid itself (Chapter 2). Thus, the requirement for the PPEA moiety on the LPS and OmpA for plasmid transfer (Section 2.2), is determined by the allelic specificity of *traN* and not by the pilus. This is congruent with Achtman's (1978) observation that ConF$^-$ recipients defective in either LPS or OmpA, were able to attach purified F pili but were severely impaired in the formation of mating aggregates. The nature of the F pilus receptor on the recipient cell still remains elusive; however, the inability of purified F pili to attach to either *Bacillus* or *Pseudomonas* cells (Achtman and Skurray, 1977) suggests that the receptor could be a component of the cell envelope unique to enteric bacteria.

Thus, due to its long flexible nature, the true role of the F pilus might be to function as a lasso in order to initiate more frequent or persistent surface contacts with cells disbursed in an aqueous environment. This first contact with a cell envelope component must be relatively non-specific since F pili adsorb to F$^-$, F$^+$ and ConF$^-$ cells (Achtman and Skurray, 1977). Following pilus retraction, the mating cells are brought into surface contact to form aggregates held together by loose association of their cell envelopes. The mating aggregate stabilization proteins, TraG and TraN, most probably act at this stage to stabilize the union perhaps by promoting membrane fusion among the conjugating cells. Direct interaction between F TraN on the outer membrane of the donor, and LPS and OmpA of the recipient cell is expected for formation of a functional or productive mating contact. Specificity in mating pair formation among F-like plasmids which determines the requirement for specific LPS moieties and OmpA, occurs at this stage. Further, interactions between TraG* and TraN in the periplasm of the donor, could bring the transfer machinery at the base of the pilus into apposition with the recipient cell, possibly at membrane adhesion zones in preparation for DNA transfer. Consistent with this proposal, mutations affecting either *traG* or *traN* prevent the formation of stable mating aggregates (Achtman and Skurray, 1977). Unlike LPS or

OmpA defects which can be overcome on solid surface matings, TraN defects are compensated only to a small extent when matings are performed on solid surfaces (Achtman and Skurray, 1977), suggesting that TraN plays an additional role beyond the recognition of LPS receptors and OmpA, most likely in association with TraG in the stabilization process.

The elaboration of an extended retractable pilus by F-like plasmids may represent an adaptation that has evolved to facilitate conjugation in the aqueous environment of enteric organisms. Likewise, the mating aggregate stabilization system may represent a complementary adjustment to such an environment. The involvement of TraG in both pilus assembly and mating aggregate stabilization is certainly consistent with this proposal. With regard to this possibility, the well-characterized non-F-like mating systems of IncP plasmids such as RP4, encode short rigid pili which naturally mediate DNA transfer on solid surfaces only (Bradley, 1983). The absence of homologues of the F TraG and TraN proteins in this plasmid suggests that surface-obligatory conjugation systems may function independently of an aggregate stabilization mechanism (Frost and Simon, 1993). Interestingly, the IncI1 plasmid, R64, which can conjugate proficiently in either liquid or on solid media, typically synthesizes two types of conjugative pili, the thin flexible and the thick rigid types (Komano *et al.*, 1995). Thick pili are essential for DNA transfer but thin pili are required only for matings in liquid medium. Mutations resulting in the loss of thin pili convert the R64 mating system from a universal to a surface-dependent mating phenotype. Thus, thin flexible pili appear to function as a mating-aggregate stabilization device that promotes conjugation in liquid medium (Frost, 1993). In addition, thin pili are important in determining recipient cell specificity via a DNA inversion mechanism which results in the alteration of the C-terminal sequences of the minor pilus protein, PilV (Komano *et al.*, 1995). A unique DNA rearrangement designated shufflon is located downstream of the *pilV* coding sequence. The shufflon consists of four DNA segments which are flanked and separated by seven 19-bp repeat sequences. Site-specific recombination between any two inverted repeats results in a complex DNA rearrangement which alters the sequence of the C-terminal segment of PilV to one of seven different combinations. Transfer frequencies of R64 in liquid medium to various *E coli*-B, C and K-12, *Salmonella typhimurium* and *Shigella flexneri* recipients

strongly depend on the combination of the C-terminal segments of *pilV* (Komano *et al.*, 1995). Thus, unlike the F plasmid where a mating aggregate stabilization system independent of the pilus seems to determine recipient cell-specificity, the R64 plasmid has evolved a pilus to perform analogous functions.

6.1.4 Role of the pilus in surface exclusion

The molecular mechanism of surface exclusion and the complementary role of the pilus in this phenomenon have long remained unresolved. Minkley and Willetts (1986) proposed a direct interaction between the pilus tip and TraT as the mechanism for TraT-mediated surface exclusion in accordance with Meynell and Ewin's (1973) proposal that the surface exclusion specificity comes about by matching the pilus with the homologous TraT protein. In contrast, Achtman proposed that surface exclusion acts at a stage beyond pilus recognition based on his observation that recipient cells expressing the F TraT protein were unable to form stable mating aggregates despite their ability to attach the F pilus (Achtman *et al*, 1980). The findings of Chapter 5 provided the genetic evidence that the pilus does not play an explicit role in defining the surface exclusion specificity among F-like plasmids. Rather, in agreement with Achtman's predictions, surface exclusion appears to act at the aggregate stabilization stage specifically where the product of *traG* is required. This was demonstrated by the transfer of F*lac traG106* in the presence of R100-1 *traG* allele with no significant surface exclusion to recipients expressing both F TraS and TraT proteins (Section 5.2). In contrast, similar experiments with an F *traN* mutant expressing the R100-1 *traN* allele revealed that the presence of either F TraT or both TraT and TraS proteins in the recipient cell significantly diminished plasmid transfer, indicating that TraN does not play a role in surface exclusion (Klimke and Frost, 1998; Klimke, unpublished results).

Although both TraT and TraS synergistically contribute to block plasmid entry, they operate through quite distinct mechanisms. As such, the precise nature of how TraG interacts with the surface exclusion proteins is not clear. However, since *traT*$^+$ but not *traS*$^+$ recipients are severely impaired in stable mating aggregate formation (Achtman *et al.*, 1977), sequence-specific interactions between TraT on the outer membrane of recipient cells, and periplasmic

TraG* in the donor cells, could conceivably interfere with the aggregate stabilization process by either preventing TraG-TraN association or the interaction of TraG* with a recipient cell receptor. Riede and Eschbach (1986) had suggested that the F TraT protein in the recipient masks an extracellular domain in OmpA that is involved in F plasmid conjugation. The apparent lack of a role for TraN in surface exclusion and the genetic evidence for TraN-OmpA interaction combined with the observation that surface exclusion is operative even on solid surface matings where the function of OmpA is dispensable, argue against this possibility. The addition of purified TraT to mating cells *in vitro* might reduce stable aggregate formation in an analogous manner (Minkley and Willetts, 1984).

Since TraS in the inner membrane of the recipient prevents the triggering of conjugal DNA metabolism in the donor cell, the interaction between TraS and TraG seems rather complex. The transmission of the mating signal occurs very early during the conjugation process most likely following pilus retraction upon contact with a recipient cell, by a mechanism which may entail an interaction between the base of the pilus and the DNA transfer complex in the cytoplasm. This process is independent of aggregate stabilization as mutations affecting neither *traN* nor *traG* prevent the signal transduction (Manning *et al.*, 1981). Since TraG could conceivably interact with TraS only when the mating cells are in intimate contact, sequence-specific interactions between TraG and TraS may function to counteract the mating signal and halt DNA processing. It is not clear, however, whether TraG in the inner membrane which might function to dock the pilus, or TraG* is required for TraS-mediated surface exclusion. Nevertheless, based on the observation that surface exclusion between cells carrying the R1 plasmid could be abolished by converting the donor cells, but not the recipients into spheroplasts (Beard and Bishop, 1975), the periplasmically located TraG* might be important for this process.

Although the molecular mechanism of surface exclusion has proven difficult to dissect, the recent finding that TraG is a key player in this process should be important in developing new approaches to understanding this process at a molecular level. Biochemical analyses with chemical cross-linking as an adjunct to immunoprecipitation using TraG-specific antisera (Firth and Skurray, 1992) would prove fruitful in identifying specific

interactions between TraG and either TraT or TraS. Thin section analysis coupled with immunogold labelling experiments of mating cells should help define the nature of the conjugational junctions described by Durrenberger (Section 1.5.1) and identify the presence of mating aggregate stabilization and surface exclusion proteins at these junctions.

6.1.5 Role of the pilus in DNA transfer

Brinton's initial hypothesis that the pilus acts as a conduit for the passage of single-stranded DNA has gradually been replaced by the more prevalent notion that the pilus acts as a grappling hook that retracts to bring the mating cells into intimate contact to form conjugational junctions through which DNA is transferred (Section 1.5.1). However, there are several reasons which suggest that the latter may represent an oversimplification of pilus function and that the pilus itself or a vestige remaining after retraction may form a channel for the translocation of DNA. Firstly, the findings of Ou and Anderson (1970) and Harrington and Rogerson (1990) that DNA transfer can occur between physically separated cells, albeit at low frequency, indicate that the lumen of the pilus can accommodate single-stranded DNA. Secondly, the apparent complexity of F pilus biogenesis orchestrated by the products of fifteen genes might reflect a multi-functional role for the pilus. Although pilin is the only protein that is associated with the pilus, mutations affecting any one of the remaining thirteen essential genes abolish pilus synthesis and DNA transfer. In contrast, only about half the number of proteins seem to be required for the synthesis of adhesive type I or P pili in *E. coli*, and a number of those are minor subunits of the pilus itself (Hultgren *et al.*, 1991). Thirdly, mutations affecting *traD*, whose product is required for the active transport of single-stranded DNA across the cell envelope, result in a slightly increased level of piliation suggesting a link between the DNA processing machinery and pilus function (Firth *et al.*, 1996). In fact, the DNA binding ability of TraD and its interaction with TraM, a component of the DNA processing machinery, have been experimentally demonstrated (Panicker and Minkley, 1992; Disque-Kochem and Dreiseikelmann, 1997). The notion that TraD might couple DNA processing to the transfer event by mediating interactions between the conjugal DNA metabolism proteins and the DNA transfer pore, has also been proposed for TraD

homologues in other transfer systems (Lanka and Wilkins, 1995). By far, the most compelling evidence for the contention that pilus components might form a mating channel comes from the extensive similarities in the characteristics between the *virB* and *tra* proteins (Section 1.7). Although the existence of a mating channel analogous to the T-complex transport machinery has not been demonstrated in the F system, the cellular distribution of the pilus assembly proteins strongly supports this notion. Structural studies to determine interactions among pilus assembly proteins are pertinent in determining the nature of the mating channel. Since a vast number of proteins are involved in this process, preliminary studies could entail identifying specific features in proteins which mediate protein-protein interactions, such as coiled-coil domains (Lupas, 1996). Analysis for the presence of such domains among the pilus assembly proteins could be performed using the readily-available COILS and MULTICOIL algorithms (Lupas *et al.*, 1991; Wolf *et al.*, 1997). Using these programs, Pallen *et al.* (1997) identified predicted coiled-coil domains in various proteins involved in macromolecular assembly including the TraC protein of the IncN conjugative plasmid, pKM101, which is the homologue of F TraE (Firth *et al.*, 1996). Identification of such coiled-coil domains should provide a framework for more rigorous experiments such as chemical cross-linking or immunoprecipitation using specific antisera to isolate the proteins that form the mating channel.

6.1.6 Bacterial conjugation: A novel macromolecular 'secretion' system?

The similarities identified among protein components involved in such diverse processes as the assembly of type IV fimbriae and flagella, extrusion of filamentous phage, DNA transfer and protein secretion, illustrate that these mechanisms are variations of a common theme (Hobbs and Mattick, 1993). The striking resemblance and colinearity among the *tra* gene products of the plasmid, pKM101, *virB* of *A. tumefaciens* and the *ptl* proteins required for the export of the pertussis toxin in *Bordetella pertussis* has been well documented. Even more provocative is the most recent finding that a conjugative apparatus is used for the delivery of virulence factors to eukaryotic cells by the intracellular pathogen, *Legionella pneumophila* (Vogel *et al.*, 1998). These close evolutionary links suggest that protein secretion

mechanisms and conjugal transfer systems which were once considered to operate on mechanistically different principles could have common ancestry (Winans *et al.*, 1996).

More recently, the mechanism of T-DNA transfer has been compared to the type III protein secretion family identified in bacterial pathogens of plants and humans (Salmond, 1994). The type III delivery system directly 'injects' protein substrates into eukaryotic cells by a process requiring cell contact and in some instances the elaboration of extracellular filaments which technically resemble conjugative pili (Christie, 1997; He, 1997; Lee, 1997). For this reason, Salmond (1994) had assigned the T-DNA transfer system and by homology, pilus-mediated DNA transfer, into the type IV secretion family. By definition, members of the type IV family involved in conjugation, transmit DNA to recipient cells by a contact-dependent process (Christie, 1997). This definition distinguishes the type IV family from other dedicated secretion pathways. These include the *sec*-independent type I system whose prototype member is the *E. coli* hemolysin; the type II system exemplified by pullulanase secretion in *Klebsiella oxytoca* where the exported molecule is moved across the inner membrane in a *sec*-dependent manner and across the outer membrane via an array of accessory proteins; and lastly, the *sec*-independent type III system for the delivery of virulence factors into host cells following direct contact as exemplified by Yop export in *Yersinia pestis* (Finlay and Falkow, 1997).

Although significant advancements have been made in our understanding of the biochemical basis underlying conjugal DNA metabolism, insights into the mechanistic basis of cell-cell DNA transfer has proven more elusive. The detection of evolutionary and functional relationships between protein secretion machinery and conjugative DNA transfer systems which highlight a role for the pilus in DNA transmission, should stimulate progress towards understanding this key biological process. Studies of the F factor will remain central to these efforts.

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APPENDIX I

CHAPTER 7

Determination of nucleotide sequence of R100-1 *traF* and production of a TraF-specific antiserum

7.1 INTRODUCTION

The *traF* locus which maps between *traN* and *traQ* encodes a product essential for pilus assembly (Frost *et al.*, 1994). The 26 kDa mature form of TraF which is derived from a 28 kDa precursor following signal sequence cleavage, is predominantly localized in the periplasm (Wu *et al.*, 1988). Like all the other pilus assembly genes, the precise function of TraF in the assembly process is not known.

During the course of the F *tra*-mutant complementation studies described in Chapter 5, the R100-1 *traF* allele appeared to alter the plating efficiency of f1 phage by cells harbouring the *traF* amber mutant, F*lac traF13* (Table 5.1). Complementation of F*lac traF13* with the R100-1 allele resulted in a phenotype that was proficient in conjugation, sensitive to R17 infection but almost resistant to f1 infection although their pili attached the phage well. These characteristics suggested that the allelic differences between F and R100-1 *traF* might determine the variation in f1 plating efficiency between these plasmids and further led to the suspicion that TraF might be a potential pilus tip adhesin.

In order to investigate this possibility, the nucleotide sequence of the *traF* alleles from both R100-1 and ColB2 plasmids were determined. The amino acid sequence of their *traF* products were compared to that of F in order to pinpoint the residues that are important for f1 infection. In addition, a TraF fusion protein tagged with 6His residues at the C-terminus was created in order to generate polyclonal TraF-specific antisera. Using immunogold electron microscopy, this antiserum was subsequently used to investigate whether TraF is localized at the pilus tip.

Prior to the completion of this work, it became evident that the earlier results obtained from the complementation studies were spurious. The drastic decrease in f1 plating efficiency by cells harbouring F*lac traF13* complemented with R100 *traF* was due to the overexpression of the protein from a high copy number plasmid and not due to allelic differences. Identical results were obtained when TraF from the F plasmid was overexpressed in a similar manner.

In this chapter, the nucleotide sequence of the R100-1 *traF* allele along with a sequence comparison of its product with its F counterpart are reported. In addition, this work

also describes the construction of a TraF-6His fusion protein and its subsequent use for the production of TraF-specific antisera.

7.2 RESULTS

7.2.1 Cloning, determination and analysis of the nucleotide sequence of R100-1 *traF*

A 1735 bp *Eco*RI-*Sst*I fragment spanning the distal end of R100-1 *traN* and the proximal end of *trbA* (corresponding to F coordinates 14212-15947, Frost *et al.*, 1994), was cloned into the high copy number plasmid, pBluescript KS+, to create the recombinant plasmid, pKAR-8, as described in Section 7.4. The nucleotide sequences of *trbE*, *traF* and the partial sequence of *trbA* were determined. The DNA sequence is presented in Fig. 7.1. Two complete ORFs corresponding to *trbE* and *traF* are shown along with the deduced ATG codons; the Shine-Dalgarno consensus sequence for *traF* is also shown. Translation initiating at these codons would give rise to 86 and 247 amino acid products with calculated molecular weights of 9 910 and 27 973 for *trbE* and *traF*, respectively. The length of the ORFs and their product-size are equivalent to their F counterparts.

7.2.2 Sequence comparison between F and R100-1 *traF* products

The pairwise sequence alignment of both F and R100-1 TraF proteins is shown in Fig. 7.2. The R100-1 TraF exhibits 98.7% overall identity with the F sequence with only three conservative substitutions in the entire protein. These changes are Ala_{87}→Thr, Arg_{100}→Lys and Ala_{131}→Gly. Like the F sequence, R100-1 TraF contains a conserved signal sequence which forms the most distinct hydrophobic segment in the entire protein. If peptide cleavage occurs between residues 19 and 20 which is the proposed cleavage site in F (Wu *et al.*, 1988), a periplasmically located mature product of approximately 25 942 molecular weight would result.

Fig. 7.1 Nucleotide sequence of R100-1 *trbE* and *traF* genes. The DNA sequence of the region encompassing *trbE* and the proximal end of *trbA*, corresponding to F coordinates 14826-15972 (Frost *et al.*, 1994), is shown. Sequences corresponding to the ribosome binding site consensus are underlined and stop codons are indicated by asterisks. ATG initiation codons are enclosed in grey boxes and the arrows indicate the direction of the transcript. The coordinates for the beginning and the end of each gene are shown in brackets adjacent to the arrow. Recognition sequences for restriction endonucleases which are also present in the F sequence are shown.

trbE (4-261)
1 GAAATGATGAAAGTTATATTCACTTCTAATCGTCTTGTTGATTTTCTTAT 50
51 CCGGCTTTCGATTACAGCGATTGTCATTTCCCCGGTAATTATCTGGTCGT 100
101 GGGATACGGTAAAAGAAACCACGGCAGACGGGATGCTTGCCGCTGCTTTC 150
151 GTTATCCTTTACTCCGGTGTGCTCCTGTTTATTCTGTATTTTTGTTTTTC 200
Pst I
201 TGCCCTGACTGGCCTGCAGAAAGATGATGAAAGAAAAAATGATGAAAGGA 250
traF (257-997)
251 ATGAAGATGAATAAAGCATTACTGCCACTGTTACTCTGCTGCTTTATTTT 300
* trbE stop
301 TCCGGCGTCAGGAAAAGATGCAGGCTGGCAGTGGTATAACGAGAAAATAA 350
Pst I
351 ATCCGAAGGAAAAAGAAAATAAACCTGTACCTGCAGCCCCCCGTCAGGAG 400
Pst I
401 CCGGATATTATGCAGAAACTGGCCGCGCTGCAGACGGCAACAAAGCGGGC 450
451 GCTGTACGAAGCCATTCTGTATCCCGGCGTGGATAATTTTGTTAAATATT 500
Pst I
501 TCCGGCTGCAGAATTACTGGACTCAGCAGGCCGGGCTTTTCACCATGAGT 550
Pst I
551 GCTAAAAAGGCCATGCTGGCACATCCCGAACTGGACTATAACCTGCAGTA 600
Pvu II
601 CAGTCATTACAACGGCACGGTACGGAACCAGCTGGCGGCAGACCAGGCGC 650
651 AGCAGCGACAGGCCATTGCGAAACTGGCTGAACACTACGGCATCATGTTC 700
701 TTTTACCGGGGGCAGGACCCCATCGACGGGCAACTGGCGCAGGTCATTAA 750
751 TGGCTTCAGGGATACCTATGGTCTGAGTGTTATTCCCGTTTCCGTGGATG 800
801 GCGTGATTAATCCGCTGTTGCCGGATTCCCGGACTGACCAGGGGCAGGCG 850
851 CAGCGCCTCGGTGTGAAATATTTCCCGGCCATGATGCTGGTTGACCCGAA 900
901 ACAGGGCAGTGTTCGCCCGTTATCATACGGCTTTATTTCACAGGACGACC 950
951 TGGCAAAACAGTTCCTGAACGTTTCTGAAGATTTTAAACCGAATTTTTAA 1000
traF stop *
1001 CCAAAGGTTATATATGCATACAGGGGATTTGTGTTTTTTTACAGGAGCCA 1050
1051 GCCTATTTATCGTGGGGGTTGTTGGTTATTTCTTTACTGGACCAAATAAT 1100
Sac I
1101 AAAGGAGTTTATCTGCTGAGCTCCAGCTTTTGTTCCCTTTAGT 1143

Fig. 7.2 Sequence alignment of F and R100-1 TraF proteins. The pairwise alignment was generated with the program GAP in the GCG package (Devereux *et al.*, 1984) using the Dayhoff 250 PAM matrix (Dayhoff *et al.*, 1978) with a gap weight of 3.0 and a gap length penalty of 0.2. The F sequence is shown below its respective R100-1 counterpart. Non homologous residues are enclosed in grey boxes.

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R100-1  1    MNKALLPLLLCCFIFPASGKDAGWQWYNEKINPKEKENKPVPAAPRQEPD   50
F       1    MNKALLPLLLCCFIFPASGKDAGWQWYNEKINPKEKENKPVPAAPRQEPD   50

       51    IMQKLAALQTATKRALYEAILYPGVDNFVKYFRLQNYWLQQAGLFTMSAK   100
       51    IMQKLAALQTATKRALYEAILYPGVDNFVKYFRLQNYWAQQAGLFTMSAR   100

      101    KAMLAHPELDYNLQYSHYNGTVRNQLAADQGQQRQAIAKLAEHYGIMFFY   150
      101    KAMLAHPELDYNLQYSHYNGTVRNQLAADQAQQRQAIAKLAEHYGIMFFY   150

      151    RGQDPIDGQLAQVINGFRDTYGLSVIPVSVDGVINPLLPDSRTDQGQAQR   200
      151    RGQDPIDGQLAQVINGFRDTYGLSVIPVSVDGVINPLLPDSRTDQGQAQR   200

      201    LGVKYFPAMMLVDPKQGSVRPLSYGFISQDDLAKQFLNVSEDFKPNF      247
      201    LGVKYFPAMMLVDPKQGSVRPLSYGFISQDDLAKQFLNVSEDFKPNF      247
```

7.2.3 Determination of ColB2 *traF* nucleotide sequence

A 760 bp fragment containing ColB2 *traF* was amplified by PCR and its sequence was determined by automated sequencing. Analysis of the DNA sequence showed 100% identity at the nucleotide level to *traF* of the F plasmid (data not shown).

7.2.4 Construction of TraF-6His fusion protein

A TraF fusion protein with a 6His tag at the C-terminus was constructed to enable the production of the protein in sufficient quantities and adequate purity for use as an immunogen. In addition to its poor-immunogenic property and low interference with the cellular function of the protein, the 6His tag affords the efficiency and convenience of purification of the fusion protein by Ni^{+}-affinity chromatography (Section 4.4).

In order to create the tagged protein, the *traF* gene from the F plasmid was amplified by PCR and was integrated into the *Nco*I and *Bam*HI sites of the expression vector, pQE-60 (Qiagen Inc.) to generate the recombinant plasmid, pQE60-F6His (Fig. 7.3A). DNA sequencing of the insert in pQE60-F6His confirmed the in-frame fusion of the *traF* coding sequence with the six codons for histidine residues encoded by the vector. This cloning strategy introduces an additional Ala adjacent to the initiating methionine in the *traF* leader sequence and as shown in Fig. 7.3B, four additional amino acids, Gly, Ser, Arg, and Ser, arising from the *Bam*HI and *Bgl*II sequences in the vector are also introduced in the mature protein upstream of the 6His residues. Thus, this gene fusion would give rise to a TraF hybrid protein containing a 4-amino acid spacer and a 6His tag at the C-terminus (Fig. 7.3B). Incorporation of the 10 additional residues would result in the synthesis of a 29 kDa precursor and a 27 kDa mature product after signal sequence cleavage.

To ensure that a TraF fusion protein of the correct molecular weight is synthesized, the *Eco*RI-*Hin*dIII fragment encoding the entire sequence of the fusion protein from pQE60-F6His was cloned into pBluescript KS+ to generate the plasmid, pBS-F6His. The coding sequence of the fusion protein in pBS-F6His is cloned downstream from the bacteriophage T7 promoter sequences of the vector. An isogenic construct, pKAF-11, which contains the wild-type coding region of *traF* was also constructed by ligating an *Eco*RV-*Sst*I fragment

Fig. 7.3 Physical map of pQE60-F6His (A) and features of TraF-6His fusion protein (B). (A) P/O upstream of the fusion protein coding sequence is the *lac* promoter and operator sequences for IPTG-inducible expression. RBS denotes the ribosome binding site. The spacer sequence was introduced for in-frame fusion of the 6His tag to the *traF* coding sequence. Amp^R and ColE1 denote ampicillin-resistance and ColE1 origin of replication, respectively. (B) Leader is the *traF* signal peptide for export of the fusion protein to the periplasm. The mature protein (TraF) contains the C-terminal extension GSRS followed by the 6His tag. N and C are the amino and carboxy termini, respectively.

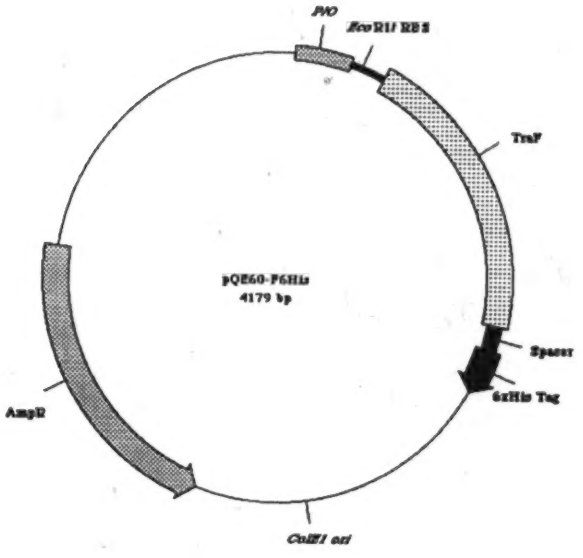


Fig. 7.3A

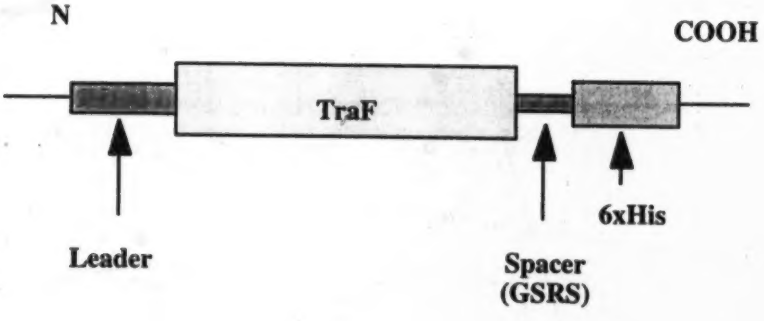


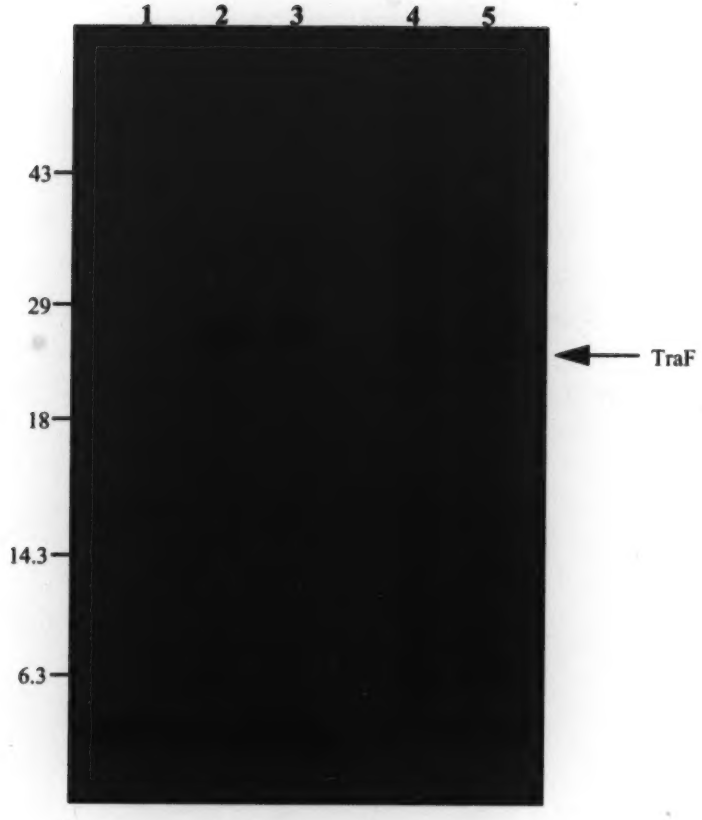
Fig. 7.3B

from the parent plasmid, pKI175, into the same vector (Table 5.2). Expression of TraF proteins from both pBS-F6His and pKAF-11 in *E. coli* XK100 cells (Section 4.4) were induced with IPTG and the proteins were labelled with [^{35}S]-methionine (Section 4.4). Total cellular proteins were analyzed by SDS-PAGE (Section 4.4) and radioactively labelled proteins were visualized by autoradiography. In the autoradiogram shown in Fig. 7.4, two distinct bands corresponding to the 29 kDa precursor and the 27 kDa mature forms of TraF-6His are visible in cells harbouring pBS-F6His (lanes 2 and 3). However, only the mature form of TraF from pKAF11 is distinctly visible at 26 kDa and the precursor form (28 kDa) is hardly detectable (lanes 4 and 5). These results show that pBS-F6His synthesizes a fusion protein of the predicted molecular weight.

7.2.5 Functional analysis of TraF-6His

Since the addition of 6His tags to proteins has been previously shown not to interfere with their normal cellular function in certain systems (Riechmann and Holliger, 1997), it was of interest to determine if TraF-6His was able to complement the *traF13* amber mutation. *E. coli* ED2149 cells harbouring the plasmid, F*lac traF13* (Tables 5.1 and 5.2), were transformed with either pKAF-11 or pBS-F6His. The ability of these chimeras to restore functional pilus production was determined by mating assays and TEM analysis (Section 2.4). The results showed that while pKAF-11 restored wild-type levels of mating efficiency and pilus production to F*lac traF13*, pBS-F6His was unable to restore any of these functions (data not shown). These results indicate that although the incorporation of additional residues at the C-terminus of TraF does not prevent the synthesis and subsequent maturation of the protein (Fig. 7.4), it prevents TraF function in pilus assembly.

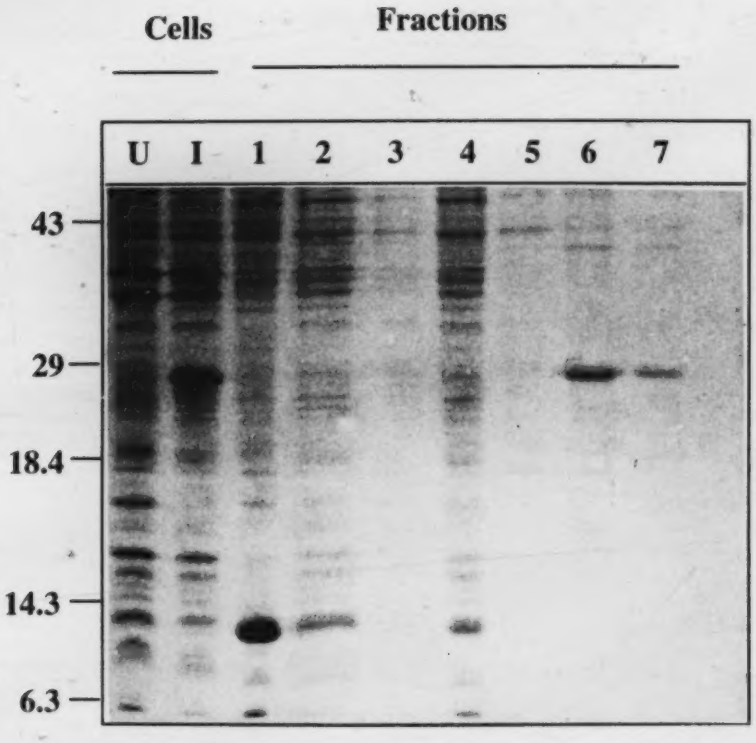
7.4. SDS-PAGE analysis of synthesis of TraF and TraF-6His. *E. coli* XK100 cells harbouring pBluescript KS+, pKAF-11 or pBS-F6His were induced with 1 mM IPTG. Plasmid-encoded proteins were radioactively labelled with [^{35}S]-methionine following the addition of rifampicin (Section 4.4). Total cellular proteins were separated on a 15% SDS-polyacrylamide gel and the proteins were visualized by autoradiography. XK100/pBluescript KS+ (lane 1), XK100/pBS-F6His (lanes 2 and 3) and XK100/pKAF-11 (lanes 4 and 5). Molecular mass markers (kDa) are shown on the left.


1
2
3
4
5
43
29
18
14.3
6.3
TraF

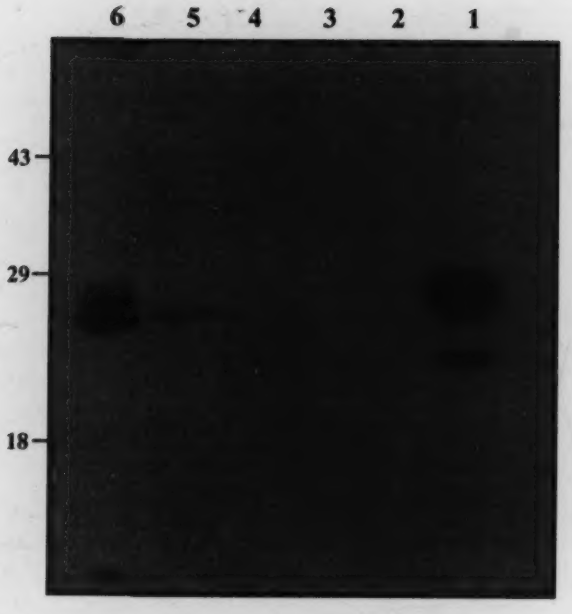
7.2.6 Overexpression and affinity purification of TraF-6His and its use as an immunogen

E. coli K802 cells were transformed with plasmids pQE60-F6His and pDMI.1; the latter overproduces the *lac* repressor (Section 4.4). After induction with IPTG, cell lysates containing soluble and insoluble proteins were prepared as described in Section 7.4. TraF-6His from both soluble and insoluble fractions was affinity purified on Ni^{2+}-NTA resin under native and denaturing conditions, respectively (Section 7.4). TraF-6His adsorbed strongly to Ni^{2+}-NTA resin and it was eluted with a gradient of imidazole (Section 7.4). Fig. 7.5 shows an SDS-polyacrylamide gel containing products of the affinity purification. A 29 kDa product corresponding to the predicted size of the unprocessed form of TraF-6His is seen only in lysates of induced cells (lane 2) and in the eluate fractions (lanes 6 and 7). The 27 kDa mature product however, is not visible. Cellular fractionation studies later revealed that only a very small proportion of the TraF-6His synthesized was processed and subsequently exported to the periplasm while the majority remained in the precursor form among the soluble and insoluble material (data not shown). As with most overexpressed proteins in *E. coli*, this is probably due to saturation of the cellular secretion machinery (Makrides, 1996). Since the choice of the immunogen whether precursor or mature protein is not critical, the precursor form was purified and was subsequently used to immunize a rabbit as described in Section 7.4. The resultant antiserum was tested for its ability to recognize TraF in Western blots. Figure 7.6 shows that the antiserum not only recognized the fusion protein that had been used as the immunogen (lane 1) but also native TraF synthesized by cells harbouring pOX38-*tra715* or pKAF11 (lanes 5 and 6). Due to the small amounts of TraF produced, the antiserum was unable to detect the protein in cells harbouring the pOX38-Km plasmid (lane 4). The specificity of the antiserum was demonstrated by the absence of a signal at the same position in the uninduced control (lane 2). However, when the antiserum was used at dilutions higher than 1:30 000, non-specific reactions with cellular proteins were observed. Thus, for future applications it is necessary to purify the antiserum further by pre-adsorption with purified TraF-6His to isolate TraF-specific antibodies.

7.5. Affinity purification of TraF-6His. *E. coli* K802 cells harbouring pQE60-F6His and pDMI.1 plasmids were induced with 1 mM IPTG for four hours. Soluble proteins were prepared as described in Section 7.4. TraF-6His in the soluble fraction was affinity purified on Ni^{2+}-NTA resin and the adsorbed protein was eluted with a gradient of imidazole in sonication buffer (Section 7.4). Total cellular proteins from uninduced and induced cultures (lanes U and I, respectively) together with the products from the affinity purification were separated on a 15% SDS-polyacrylamide gel. The proteins were visualized by staining with Coomassie Brilliant Blue. The lanes correspond to the unbound fraction (1), wash fractions with 10 mM, 30 mM, 40 mM and 50 mM imidazole (2-5) and eluate fractions with 500 mM imidazole and 50 mM EDTA (6 and 7). Molecular mass markers (kDa) are shown on the left.


Cells
Fractions
U
I
1
2
3
4
5
6
7
43
29
18.4
14.3
6.3

7.6 Western blot analysis of products expressed by cells containing *traF*-encoding plasmids using TraF-specific antisera. Total cellular proteins were prepared as described in Section 4.4. Proteins were fractionated on a 15% SDS-polyacrylamide gel before being transferred to a PVDF membrane for Western blot analysis (Section 4.4). TraF was detected immunologically using TraF-specific antisera (1 in 30 000 dilution) and goat anti-rabbit HRP conjugate (1 in 2000). Lanes: 1, pQE60-F6His/pDMI.1/K802 (induced); 2, pQE60-F6His/pDMI.1/K802 (uninduced); 3, F*lac traF13*/ED2149; 4, pOX38-Km/MC4100; 5, pOX38-*tra715*/XK100; 6, pKAF-11/XK100. Molecular mass markers (kDa) are shown on the left.


6
5
4
3
2
1
43
29
18

7.3 DISCUSSION

This study entailed the determination of the nucleotide sequences of R100-1 *trbE* and *traF* genes. Sequence comparisons revealed that the R100-1 *traF* product is remarkably identical to F TraF with only three conservative substitutions in the entire protein. In addition, a TraF fusion protein with a 6His tag at its C-terminus was constructed and polyclonal TraF-specific antiserum was produced using the fusion protein as the immunogen. The loss of cellular function of TraF-6His suggests that the C-terminus is important in its function in pilus assembly.

Although the specific role of TraF in pilus assembly is still unknown, its periplasmic location suggests that it might be a structural component of the conjugation apparatus (Section 1.7.1). As such, interactions between TraF and other periplasmic pilus assembly proteins such as TraK, H and W or the outer membrane, TraV, is conceivable. So far, attempts to demonstrate protein-protein interactions during pilus assembly have never been undertaken due to lack of specific antisera or other means of identifying these proteins. To this end, the TraF-specific antiserum together with the pOX38-*tra715* expression system should greatly aid in the identification of interactions between TraF and other transfer proteins and set a precedent for the beginning of a systematic analysis of the function of pilus assembly proteins during the formation of the conjugation apparatus. The finding that overexpression of TraF drastically decreases the susceptibility of an F^+ cell specifically to f1 phage infection without compromising its ability to plate R17 phages or transfer DNA, is intriguing. Although the role of TraF in this process is not clear, it is tempting to speculate that increased amounts of TraF in the periplasm might interfere with the function of *tol* gene products which are required for phage uptake (Section 1.6.2; Webster, 1996). Interactions between TraF and either TolA or TolR should also be investigated in future studies.

7.4 MATERIALS AND METHODS

7.4.1 Bacterial strains, plasmids and general molecular biology techniques

E. coli, K802, XK100 and ED2149 and plasmids, pDMI.1, pOX38-*tra715* and F*lac traF13* have been described previously in Sections 4.4 and 5.4. The procedures for bacterial transformation, recombinant DNA and PCR are described in Section 2.4.

7.4.2 Cloning and nucleotide sequence determination of R100-1 *traF*

A 1735 bp *Eco*RI-*Sst*I fragment carrying the R100-1 *trbE* and *traF* genes (corresponding to F coordinates 14212-15947) was excised from the parent plasmid, pBK-7 (Section 5.4), and was ligated into the *Eco*RI-*Sac*I sites of pBluescript KS+ to create pKAR-8. The nucleotide sequence of both *trbE* and *traF* from pKAR-8 was determined by dideoxy sequencing (Section 3.4) using the following four primers: KAF-1 (5'-GTATGATAACGGGCGAACACTG-3'), KAF-2 (5'-CGGGAATAACACTCAGACCA-3'), KAF-3 (5'-CTGCCATGGCTAATAAAGCATTACTGCC-3') and KAF-4 (5'-CGCGGATCCAAAATTGGGTTTAAAATC-3').

7.4.3 Determination of the nucleotide sequence of ColB2 *traF*

The ColB2 *traF* gene was amplified by PCR using ColB2 plasmid as the template and the primers KAF-1 and KAF-2. The nucleotide sequence of the 760 bp fragment was determined by automated sequencing using the same four primers as above.

7.4.4 Analysis of DNA and protein sequences

Analysis of both nucleotide and deduced amino acid sequences of *traF* was done using the GCG package (Devereux *et al.*, 1984). Pairwise sequence alignments were generated with the program GAP (Devereux *et al.*, 1984) using the Dayhoff 250 PAM matrix (Dayhoff *et al.*, 1978) with a gap weight of 3.0 and a gap length penalty of 0.2.

7.4.5 Construction of TraF-6His fusion protein

Addition of the six histidine tag to the C-terminus of TraF was achieved by cloning the gene into the expression vector pQE-60 (Qiagen Inc.). The *traF* gene from the F plasmid (coordinates 15082-15822, Frost *et al.*, 1994) was amplified by PCR using the plasmid, pRS29 (Table 5.2), as the template and primers KAF-3 (5'-CTGCCATGGCTAATAAAGCATTACTGCC-3') and KAF-4 (5'-CGCGGATCCAAAATTGGGTTTAAAATC-3'). Primers KAF-3 and KAF-4 contain *Nco*I and *Bam*HI restrictions sites respectively (underlined bases), to facilitate in-frame fusion with the 6 histidine residues in the vector. This cloning strategy also introduces an additional alanine residue adjacent to the initiating methionine in the *traF* leader sequence. The 757 bp amplified fragment was purified from agarose gel, restricted with *Nco*I and *Bam*HI enzymes and was ligated with the pQE-60 vector, to give rise to the chimeric plasmid, pQE60-F6His. In order to confirm the absence of any PCR-induced mutations as well as the incorporation of 6His tag, the sequence of the *traF* in the chimeric plasmid was confirmed by DNA sequencing.

7.4.6 Expression and purification of TraF-6His fusion protein

The plasmid, pQE60-F6His, was transformed into *E. coli* K802 cells carrying the coresident plasmid, pDMI.1 (Section 4.4). Transformants were selected on LB agar containing 50 μg/ml ampicillin and 25 μg/ml kanamycin. Ten colonies were individually inoculated in 2 ml LB broth containing ampicillin, kanamycin and 100 mM glucose. The cultures were grown at 30°C at 260 rpm until OD_{600} reached 0.8-1.0. IPTG was added to a final concentration of 1 mM to induce expression of the fusion protein for 1 hour. Total cellular proteins from uninduced and induced cultures were prepared as described in Section 4.4 and were analyzed by SDS-PAGE (Section 4.4) to determine the amount of fusion protein produced by each colony.

For large scale protein purification, 5 ml of an overnight inoculum of the colony producing the highest amount of fusion protein was inoculated into 1l LB containing ampicillin, kanamycin and 100 mM glucose. The culture was grown under the same

conditions as above except following the addition of IPTG, the culture was transferred to RT for four hours. Under these conditions, only a fraction of the protein was being exported to the periplasm; about 50% was in the soluble form while an equal amount remained insoluble. In order to obtain large quantities of the protein, both soluble and insoluble fractions were isolated as described below.

Soluble proteins were isolated by resuspending the cell pellet in 100 ml sonication buffer (50 mM $NaH_2PO_4.H_2O$ pH 8.0, 300 mM NaCl) containing 1 mg/ml lysozyme. Following incubation on ice for 30 min, the cells were lysed by sonication (small probe; 2 min burst/2 min cooling cycle; 200-300 Watt) for 10 min on ice. Ten µg/ml RNase A and 5 µg/ml DNase I were added and the mixture was incubated on ice for 15 min to remove the nucleic acid content. Insoluble material was separated by centrifugation at 12 100 g for 30 min at 4°C and the pellet containing the insoluble proteins was kept aside for later use. The supernatant containing the soluble proteins was re-centrifuged at 30 000 rpm in SW 50.1 Ti rotor for 30 min at 4°C to remove any particulate material. Imidazole was added to the supernatant to a final concentration of 10 mM. TraF-6His from the soluble fraction was affinity purified on Ni^{2+}-NTA resin as described in Section 4.4 except the following imidazole gradients were used: successive washes were done in sonication buffer containing 10, 30, 40 and 50 mM imidazole and elution was done twice with sonication buffer containing 500 mM imidazole and PBS containing 50 mM EDTA pH 6.5, respectively.

TraF-6His from the insoluble material was purified under denaturing conditions. The pellet from above was resuspended in 20 ml Buffer A (6 M GuHCl, 0.1 M $NaH_2PO_4.H_2O$, 0.01 M Tris/HCl, pH 8.0) and the proteins were denatured by stirring the mixture with a stir-bar for 1 hour at RT. After centrifugation at 12 100 g for 30 min at RT, the supernatant was re-centrifuged at 30 000 rpm in SW 50.1 Ti rotor for 30 min at RT. Five ml Ni^{2+}-NTA resin pre-equilibrated in Buffer A, was mixed with the supernatant. The mixture was gently rocked on a platform at RT overnight. After removal of the unbound material by centrifugation, the resin was washed successively with 10 volumes each of Buffer A, Buffer B (8 M urea, 0.1 M $NaH_2PO_4.H_2O$, 0.01 M Tris/HCl, pH 8.0) and Buffer C (8 M urea, 0.1 M $NaH_2PO_4.H_2O$, 0.01 M Tris/HCl, pH 6.3). The adsorbed TraF-6His was eluted from the resin by washing with 10

volumes of Buffer C containing 500 mM imidazole. The eluate was dialyzed extensively against MilliQ-H_2O, dried under vacuum and was analyzed by SDS-PAGE. The procedures described here were adapted from the 'QIAexpressionist' handbook (QIAGEN Inc.) and were modified to suit this particular application.

7.4.7 Preparation of protein samples for immunization and generation of polyclonal TraF-specific rabbit antisera

Approximately 200 µg of either soluble or insoluble affinity purified TraF-6His fusion protein, as judged by comparison with protein molecular weight markers, was separated on a 15% SDS-polyacrylamide gel. The fusion protein was visualized by staining the gel for 30 min with 0.05% Coomassie Brilliant Blue dissolved in Milli-QH_2O (Harlow and Laine, 1988). After destaining by rinsing with copious amounts of Milli-QH_2O, the light green region of the gel containing the fusion protein was carefully excised with a scalpel, taking caution to avoid extraneous material. The gel slab was finely sliced and later crushed in a sterile mortar and a pestle in 1 ml sterile PBS pH 7.3. This suspension was repeatedly passed through two luer-lock syringes connected by a metal 'bridge' to create particulate sized material that could freely pass through a 21-gauge needle. The volume of this suspension was re-adjusted to 1 ml with sterile PBS pH 7.3. This mixture was emulsified with an equal volume of Freund's Complete Adjuvant (Harlow and Laine, 1988) by repeatedly passing the suspension through the luer-lock syringes. A New Zealand White rabbit was immunized by intradermal and intramuscular injections of 0.75 ml of emulsion. Three weeks after the initial immunization, the rabbit was boosted with a repeat dose. The immunogen was prepared in an identical manner for subsequent injections except Freund's Incomplete Adjuvant was used. Each boost was performed at a minimum of 21-day interval to a total of six. The rabbit was subsequently bled after 17 weeks and the serum obtained was treated as described in Harlow and Laine (1988). Various dilutions of the serum were used to detect TraF on Western blots (Section 4.4).

7.5 REFERENCES

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